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Propagation of Measles Virus in Human Carcinoma Cells. (22643)

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(Introduced by J. F. Enders)
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Because they could not regularly be reproduced, early claims of the propagation of measles virus in tissue cultures have not met with wide acceptance. However, Enders and Peebles(1) reported that an infectious agent could be isolated from the throat or blood of measles patients and readily grown in cultures of human kidney cells.

It seemed desirable to find other cells in which the virus may be propagated since human kidneys are not readily available and monkey kidney cells, although susceptible, frequently carry a latent agent which may cause lesions similar to those of measles virus. To this end, Dr. George Foley put at our disposal the KB strain of human epidermoid carcinoma cells, originally isolated by Dr. Harry Eagle(2). These cells grow profusely in a relatively simple and well defined medium containing 10% horse serum(3,4).

In tubes to which a suspension of these cells were added, confluent sheets of growth were seen after 2 or 3 days. At this time, the cells were inoculated with the fluid from the 23rd passage in human kidney cell cultures of a strain (Edmonston) of measles virus(1).

Daily microscopic examination of the cultures revealed on the 4th or 5th day, 6 to 10 areas of "giant cell" or syncytial formation comparable to those described by Enders and Peebles(1) in cultures of human renal cells. By the following day, however, these "lesions" had fallen off the glass and the clear area was quickly overgrown by cells of normal appearance. Two to 3 days later, (Fig. 1), a second but larger group of giant cells appeared both in the same areas and elsewhere throughout the cell sheet. This process of simultaneous cell destruction and multiplication continued until complete destruction of the culture by the virus at the end of 5 weeks.

During this period, culture fluid from the fast growing yet infected primary culture was used repeatedly to start subcultures in which the same changes were observed and in which the cells underwent the same fate as those of the primary culture within about the same period. Only in the final stages were acidophilic inclusion bodies seen in several of the cells that were comparable to those described as occurring abundantly in normal human and monkey renal cells(1). Further evidence that these lesions were caused by measles virus was afforded by the specific fixation of complement using as antigens fluids from infected cultures in the presence of measles convalescent serum. Only those fluids, however, taken from cultures that were inoculated with the virus 3 to 4 weeks previously were shown to fix complement under these conditions.

From these observations it is concluded that the measles virus may be propagated in

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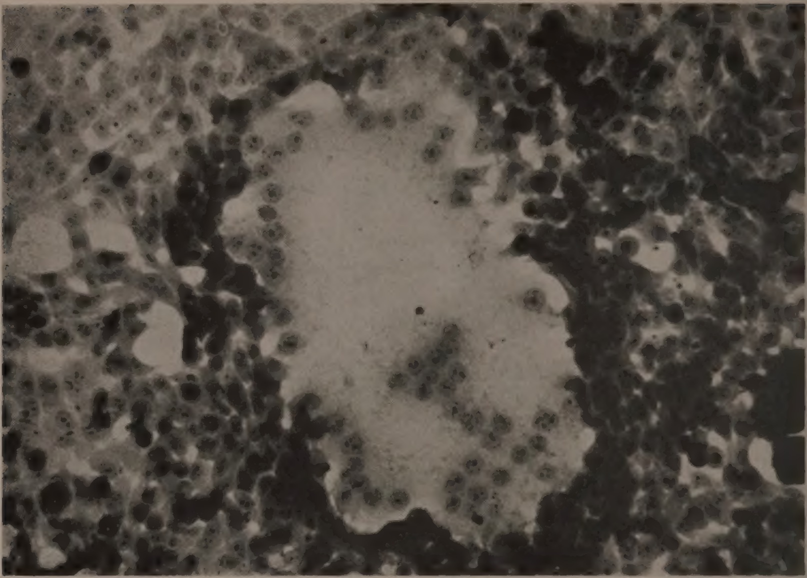

FIG. 1.

the KB strain of human carcinoma cells.

1. Enders, J. F., and Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.
2. Eagle, H., *ibid.*, 1955, v89, 362.
3. ———, *J. Exp. Med.*, 1955, v102, 37.
4. ———, *ibid.*, 1955, v102, 595.

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Lipid Metabolism in Kidney Undergoing Compensatory Hypertrophy.* (22644)

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Previous studies involving "regenerating" liver in rats(1), and the prostate and seminal vesicle tissue of non-castrated and castrated rats with and without testosterone treatment (2), suggested that there was an increased P^{32} uptake in all of the Schmidt-Thannhauser (3) fractions that was associated with cellular hypertrophy. Neither of these conditions can be considered as a hypertrophy, pure and simple, however, since the hypertrophy seen in the first 24 hours in regenerating liver is, in a sense, a preparative state for an ensuing cellular hyperplasia in that it occurs prior to the appearance of mitosis, while that in the prostates and seminal vesicles occurred in the

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† Taken in part from a dissertation submitted by Elinor Levin for the partial fulfillment of the degree of Doctor of Philosophy in Physiological Chemistry from Wayne University Graduate School.

presence of actively dividing cells. It was desirable to study the phosphorus metabolism associated with a pure hypertrophy in order to determine whether the changes observed to occur during cell growth were related to or were independent of mitosis.

When one kidney of an experimental animal is surgically removed, the remaining kidney is observed to undergo compensatory hypertrophy(4,5). In this case the complicating factors mentioned above appear to be absent or greatly minimized, and thus this tissue presents itself as one which might profitably be studied.

Experimental. Six male Fisher strain rats, 3-4 months old, were used in each experimental group. The animals were maintained on a stock diet of Purina laboratory chow and water *ad libitum* before and after unilateral nephrectomy. Nephrectomy was accomplished by making a lateral incision about ¾ inches long on the left flank, just below the inferior border of the rib cage. The kidney was raised from the incision and the adrenal gland stripped away and pushed back into the cavity. The pedicle of the kidney was ligated, the kidney excised, and the incision closed in 2 layers.

Preliminary experiments(6) were carried out to determine the percent increase of the intact kidney weight over that of the excised kidney at various times following nephrectomy. The results, calculated on both a moist and dry basis, showed that the greatest increase in weight of the intact kidney occurred about the third day, and rate of increase leveled off at about the seventh day. Therefore, the animals were sacrificed either 3 or 7 days following nephrectomy. Kidneys taken from the right side of non-nephrectomized animals served as controls.

Four hours after giving each rat a subcutaneous injection of 0.45 μc of radioactive phosphorus‡ per g body weight the animal was sacrificed. The abdominal cavity was exposed, and the intact kidney was perfused with ice cold physiological saline *in situ* via the inferior vena cava. The kidney was removed, cut into small pieces, and homogenized in 10% trichloroacetic acid containing 0.4 M $MgCl_2$(7). The phosphorus-containing fractions shown in Table I were then prepared by methods previously described(8,9, 10).

One kidney did not provide enough tissue for the fractionation described above and also for unsaturated fatty acid determinations. Therefore, additional groups of control and experimental animals were prepared in a manner identical to that described, and dienoic. trienoic, tetraenoic, and pentaenoic acids were determined on the perfused kidneys following their homogenization in physiological saline (11).

Results. Three days after nephrectomy the intact kidneys weighed an average of 10% more than those removed; after 7 days they weighed 23% more, and after 20 days they were 27% heavier than the controls. Increases in the amount of cephalin and lecithin in the hypertrophying kidneys were usually apparent 7 days after nephrectomy. However, there was a decreased P^{32} uptake by these 2 phospholipids 3 days after nephrectomy, that had returned to the normal (control) levels by 7 days (Table I). No changes in either amounts of phosphorus or of P^{32} uptake by any of the remaining fractions studied were observed either 3 or 7 days after unilateral nephrectomy (Table I). These results, especially those concerning P^{32} uptake by the phospholipid and acid-soluble fractions, were in contrast to those seen in the experiments involving hypertrophying liver 24 hours after partial hepatectomy(1) and seminal vesicles and prostates of castrated rats following testosterone treatment(2).

Samples of all tissues used in the study were fixed in Bouin's solution and prepared for histological examination(1). The mitotic activity of the kidney 3 and 7 days after nephrectomy was no greater than that seen in organs taken from the unoperated controls. While not enough is known to postulate that there are biochemically distinct types of hypertrophy, it seems possible tentatively to hypothesize as follows: If a tissue is subjected to a stimulus capable of eliciting cellu-

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‡ The radioactive phosphorus used in these experiments was furnished by the Oak Ridge National Laboratory, Oak Ridge, Tenn.

TABLE I. P^{32} Uptake and Concentration of Phosphorus in Rat Kidney Undergoing Compensatory Hypertrophy.

	Controls		3-day nephrectomy		7-day nephrectomy	
Fractions	P^{32} uptake*	μg P/mg N	P^{32} uptake	μg P/mg N	P^{32} uptake	μg P/mg N
Acid-soluble	1706 (79)†	26.7 (1.3)	1604 (70)	25.7 (.8)	1776 (58)	30.2 (.9)
Total phospholipids	488 (18)	29.5 (.8)	501 (15)	30.3 (1.0)	519 (23)	33.1 (.4)
Cephalin	333 (14)	14.1 (.4)	284 (10)	12.5 (.2)	346 (20)	16.1 (.3)
Lecithin	809 (52)	11.5 (.4)	780 (21)	13.0 (.4)	877 (90)	12.9 (.3)
Sphingomyelin	93 (4)	3.6 (.1)	87 (3)	3.5 (.2)	99 (4)	3.6 (.1)

* Figures for P^{32} uptake are concentration coefficients calculated as follows:

$$\frac{\text{Counts/min. in fraction/}\mu\text{g phosphorus in fraction}}{\text{Counts/min. injected/}\mu\text{g of body wt}}$$

† Figures within parentheses are stand. errors of mean values.

lar growth (hypertrophy) that is accompanied or followed closely by cellular increase (hyperplasia), it may be expected to show an increased P^{32} uptake in the fractions isolated by the Schmidt-Thannhauser procedure. If, however, the stimulus elicits cell hypertrophy only, it need not produce a change in the rate of uptake of P^{32} in these fractions.

The changes in the concentrations of the unsaturated fatty acids were greater in the presence of compensatory hypertrophy of kidneys, seen in the present experiments, than were observed in the case of liver hypertrophy following partial hepatectomy(12). The dienoic and tetraenoic acid contents were increased, and the trienoic and pentaenoic acids decreased 3 days after nephrectomy (Table II). By the seventh day the amounts had essentially returned to normal. The changes observed then, seemed to be associated with a cell that was rapidly enlarging in size at 3 days, rather than with the enlarged cell itself (at seven days). It is of interest, and perhaps significant, that the compensatory response in the intact kidney 3 days after nephrectomy, whatever its nature, was associated with an increase in only those fatty acids essential for growth, *i.e.*, dienoic and tetraenoic acids, and a decrease in trienoic and pentaenoic acids.

TABLE II. Concentration of Unsaturated Fatty Acids in Rat Kidneys Undergoing Compensatory Hypertrophy.*

	Control (4 animals)	3-day nephrectomy (6 animals)	7-day nephrectomy (4 animals)
Dienoic	94 (4)	112 (6)	86 (2)
Trienoic	16 (4)	4 (2)	9 (2)
Tetraenoic	123 (2)	137 (2)	109 (2)
Pentaenoic	22 (3)	14 (1)	16 (1)

* μg of unsaturated fatty acid/mg nitrogen. Figures in parentheses are stand. errors of the mean values.

Summary. The increased P^{32} uptake of the various phosphorus-containing fractions during hypertrophy in liver and prostatic tissue is not exhibited during renal hypertrophy. It is suggested that the P^{32} increases seen in the liver and prostatic tissue during hypertrophy are associated with subsequent mitotic activity found in these tissues.

1. Johnson, R. M., and Albert, S., *Arch. Biochem. and Biophys.*, 1952, v35, 340.

2. Levin, Elinor, Albert, S., and Johnson, Ralph M., *ibid.*, 1955, v56, 59.

3. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1945, v161, 83.

4. Jackson, C. M., and Shiels, M., *Anat. Rec.*, 1927, v36, 221.

5. MacKay, E. M., MacKay, L. L., and Addis, T., *J. Exp. Med.*, 1932, v56, 255.

6. Levin, Elinor, Ph.D. Thesis, Wayne University, 1955.

7. Johnson, R. M., and Dutch, P. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 662.

8. Albert, S., Johnson, R. M., and Cohan, M. S., *Cancer Research*, 1951, v11, 772.

9. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, 1948, v176, 193.

10. Ma, T. S., and Zuazaga, G., *Ind. Eng. Chem., Anal. Ed.*, 1942, v14, 280.

11. Klein, P. D., and Johnson, R. M., *Arch. Biochem. and Biophys.*, 1954, v48, 172.

12. Johnson, Ralph M., Levin, Elinor, and Albert, S., *ibid.*, 1954, v51, 170.

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Conversion of C^{14}-Arterenol to Epinephrine *in vivo*.* (22645)

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William G. Clark.

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Considerable progress has been made in elucidating the pathway of epinephrine biosynthesis by tracer studies. It has been demonstrated that phenylalanine and tyrosine are precursors of epinephrine *in vivo*(1,2) and *in vitro*(3). Adrenal epinephrine has been obtained after injection of C^{14}-3,4-dihydroxyphenylalanine and C^{14}-3,4-dihydroxyphenethylamine into rats(4). Injection into rats of C^{14}-methionine labelled in the methyl group produced C^{14}-epinephrine in the adrenal gland(5). Although indirect evidence has been offered(6,7,8), the main evidence for the conversion of arterenol to epinephrine is based on the experiments of Bülbring(9), who used bioassays to measure the increase of epinephrine in adrenal preparations incubated with arterenol and methyl donor *in vitro*. Several attempts to repeat Bülbring's experiments in this laboratory gave negative results. The present paper reports a search for C^{14}-epinephrine in the adrenals of rats after the intraperitoneal injection of C^{14}-arterenol.

Materials and methods. The α-C^{14}-dl-arterenol hydrochloride was synthesized by Howton, Mead, and Clark(10). Its activity was 16 mc per millimole. The sample had deteriorated somewhat on storage and in some experiments was purified just before use by ion exchange chromatography. A solution of 8 mg of the impure C^{14}-arterenol in 8 ml water was passed onto a column (24 cm long, 2.4 cm O.D.) of Dowex 50 resin (200-400 mesh, 8% crosslinked) in the hydrogen form in 0.5 N HCl. The column was developed with 0.5 N HCl, and fractions of approximately 20 ml were collected. The arterenol fractions (#83-107) were combined, passed through a column of 250 g Dowex 1 resin in the acetate form (1.5 times the theoretical amount) to remove the chloride ions, and lyophilized. Its purity was determined by chromatography on Whatman #1 paper using as developing solvent phenol-0.1 N HCl (85:15 (wt./vol.)) saturated with SO_2. The chromatogram was cut into segments and the radioactivity measured in a windowless flow counter (Fig. 1).

Four adult male Long-Evans rats were injected intraperitoneally with α-C^{14}-dl-arterenol hydrochloride twice a day for 3 days and sacrificed under nembutal anesthesia 24 hours after the last injection (Exp. 1 and 2; 2 rats per experiment). Six other rats were given 6 injections at hourly intervals and sacrificed one hour after the last injection (Exp. 3, 4 and 5; 2 rats per experiment). The adrenal glands were removed and kept frozen until used. Adrenals of 2 rats were ground in a glass homogenizer with 2 N acetic acid. This suspension was divided into 2 equal parts. One part was used for paper chromatography. It was evaporated *in vacuo* at 45°-50° C., and the residue extracted with several portions of ethanol (total volume, 1.0 ml.) and centrifuged. The supernatant was evaporated to less than 0.5 ml with a stream of nitrogen and the entire solution spotted on Whatman #1 paper. The chromatogram was developed overnight in the phenol solvent, washed with benzene, dried, sprayed with $K_3Fe(CN)_6$ solution(11), and counted as above.

The other half of the suspension was used for the determination of specific activity after clarifying by adjusting to pH 6.5 with 2 N NH_4OH and centrifuging. An aliquot of the supernatant was assayed for epinephrine by a modified Weil-Malherbe procedure‡(12). To the rest of the extract 15.0 mg of unlabelled l-epinephrine (as 27.3 mg of the syn-

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† Research Fellow of the Los Angeles County Heart Assn., 1955-56.

‡ We wish to thank Dr. Samuel Eiduson for the epinephrine determinations.

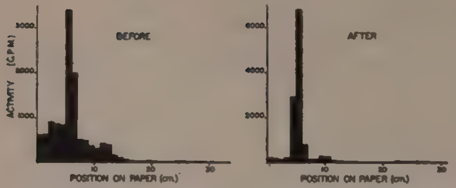

FIG. 1. Distribution of radioactivity on paper chromatogram of α-C^{14}-dl-arterenol before and after ion exchange resin treatment.

thetic bitartrate) was added as carrier. The l-epinephrine was crystallized by adjusting the solution to pH 8.8 with 2 *N* NH_4OH. The precipitate was filtered with suction, washed 3 times with cold water and once with ethanol, plated out, and counted. It was found impossible to separate epinephrine from contaminating radioactive arterenol in control experiments by a few crystallizations (also reported by Udenfriend and Wyngaarden(4)), so a method was devised which removed arterenol by virtue of its reactivity with pyridoxal-5-phosphate(13) (Table I). Sodium pyridoxal-5-phosphate solution was prepared from 1 mg of the calcium salt§ (approximately 60% pure) in 0.1 ml of 0.5 *M* phosphate buffer at pH 6.8. The epinephrine sample, which had been redissolved in acetic acid and adjusted to pH 6.8, was allowed to react with the sodium pyridoxal-5-phosphate solution for 30 minutes at 45°C. Then the epinephrine was crystallized, filtered, washed, plated, and counted as before. A second incubation and crystallization was usually sufficient to obtain constant activity.

TABLE I. Separation of Labelled Arterenol from Carrier Epinephrine in Control Experiments.

			c.p.m./mg	% arterenol in epinephrine
A.	Original mixture		8100	.40
	Crystallized	1×	6800	.34
	″	2×	5500	.27
	″	3×	4800	.24
	Pyridoxal-5-phosphate	1×	325	.016
B.	Original mixture		67	.033
	Crystallized	1×	42	.021
	Pyridoxal-5-phosphate	1×	3.4	.0017
	″ ″	2×	1.1	.0006

TABLE II. Conversion of C^{14}-Arterenol to Epinephrine.

Exp. No.	Hr	Total counts injected	Adrenal epinephrine + carrier (total c.p.m.)	Specific activity of adrenal epinephrine (c.p.m./μM)
1	72	6×10^7	683	
2	72†	6×10^7	955	
3	6	2×10^7	239	4,994
4*	6	2×10^7	383	23,728
5*	6	2×10^7	285	11,463

* Arterenol purified by ion exchange resin treatment.

† Marsilid, 100 mg/kg inj. daily.

Results. It can be seen from Table II that radioactive arterenol injected into rats was converted to adrenal epinephrine. Significant labelling of epinephrine occurred even during the 6-hour experiments. The chromatographic evidence for the presence of radioactive epinephrine is illustrated in Fig. 2, which shows the distribution of radioactivity in a typical paper chromatogram of the adrenal extract (Exp. 5). The chromatogram had been sprayed before counting and the positions of highest activity were found to correspond exactly with the colored spots due to endogenous amines.

As described by Howton, *et al.*(10), a contaminant in the radioactive arterenol has an R_F corresponding to 3, 4-dihydroxyphenethylamine. The possibility that this contaminant may have been responsible for the radioactive epinephrine in the adrenal gland does not appear to be likely, since the specific activities of adrenal epinephrine in Exp. 4 and

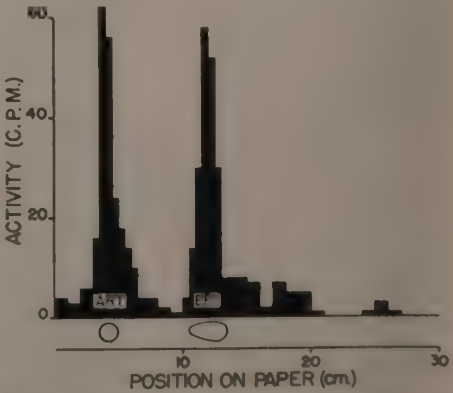

FIG. 2. Distribution of radioactivity on paper chromatogram of adrenal extract from rat inj. intraper. with α-C^{14}-dl-arterenol.

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§ Obtained from the Merck Institute for Therapeutic Research, Rahway, N. J.

5 are higher than in Exp. 3 even though the contaminant was almost completely removed in the purification of the arterenol.‖

These experiments represent the first direct evidence that arterenol is converted to epinephrine and completes the reaction sequence postulated to lead from phenylalanine to epinephrine.

Summary. C^{14}-epinephrine has been found in the adrenal glands of rats administered α-C^{14}-arterenol intraperitoneally. Its presence has been demonstrated by paper chromatography and by isotope dilution using a new method, involving reaction with pyridoxal-5-phosphate, for separating these catecholamines.

‖ We wish to thank Dr. William Drell who developed this method and demonstrated that it completely separated arterenol from epinephrine and 3,4-dihydroxyphenethylamine. Furthermore, a paper chromatogram of carrier 3,4-dihydroxyphenethylamine hydrochloride which had been recrystallized from a solution containing the purified C^{14}-arterenol hydrochloride showed no radioactivity at the R_F of the carrier.

1. Gurin, S., and Delluva, A. M., *J. Biol. Chem.*, 1947, v170, 545.

2. Udenfriend, S., Cooper, J. R., Clark, C. T., and Baer, J. E., *Science*, 1953, v117, 663.

3. Kirshner, N., and Goodall, McC., *Fed. Proc.*, 1956, v15, 110.

4. Udenfriend, S., and Wyngaarden, J. B., *Bioch. Biophys. Acta*, 1956, v20, 48.

5. Keller, E. B., Boissonnas, R. A., and du Vigneaud, V., *J. Biol. Chem.*, 1950, v183, 627.

6. Forst, A. W., and Deininger, R., *Arch. exp. Path. u. Pharmakol.*, 1952, v215, 378.

7. Engelhardt, A., Greeff, K., Holtz, P., and v. Luecken, R., *ibid.*, 1954, v221, 506.

8. Giertz, H., and Iurna, I., *ibid.*, 1955, v224, 295.

9. Bülbring, E., *Brit. J. Pharmacol.*, 1949, v4, 234.

10. Howton, D. R., Mead, J. F, and Clark, W. G., *J. Am. Chem. Soc.*, 1955, v77, 2896.

11. James, W. O., *Nature*, 1948, v161, 851.

12. Weil-Malherbe, H., and Bone, A. D., *Biochem. J.*, 1952, v51, 311.

13. Schott, H. F., and Clark, W. G., *J. Biol. Chem.*, 1952, v196, 449.

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Investigations on Polyvinylpyrrolidone in Mice Given Brucella Endotoxin.

(22646)

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Studies in these laboratories with endotoxins prepared from various species of Gram-negative bacteria, including brucellae, have confirmed the observations that these endotoxins cause profound vascular disturbances in animals, terminating in collapse and death (1,2). More precise measurements with non-lethal doses of brucella endotoxin have shown that the daily temperature rhythm of mice is affected by small amounts of the endotoxin (3). The lethal effects of endotoxin in mice can be prevented by administration of adrenal corticosteroids or chlorpromazine, and tolerance to lethal doses of endotoxin can be produced by administration of sublethal amounts of heterologous endotoxins(1,4,5,6). The hypothermia of mice given brucella endotoxin also can be counteracted with added corticosteroid(7).

The present studies were undertaken to determine if the polymer, polyvinylpyrrolidone (PVP), would protect mice against brucella endotoxin. Originally introduced as a plasma expander by Hecht and Weese(8), interest turned to using PVP as a "detoxifying" agent. Toxic substances not usually excreted in the urine were reported to be "adsorbed" to PVP and excreted as such(9,10,11). PVP not only protected animals, but it was described as also effective in the treatment of human cases of tetanus and of diphtheria(12,13,14). According to these investigators, the most effective form of PVP was incorporated in the preparation known as Periston N (PVPN), having a smaller molecular weight than the original PVP (molecular weights: PVP~ 100,000, PVPN~12,600). More recently in a therapeutic trial on 10 patients with severe tetanus, no benefit attributable to PVPN was noted(15). Likewise, in mice PVPN given 3 hours after tetanus toxin was ineffective, but it protected when given within one hour after

TABLE I. Statistical Analysis of Data Presented in Fig. 1.

	Mortality rates compared				
Comparison No.	Stock	Time, hr	PVPN*	X^2	P
1	ABC	24	0	13.73	<.01
	ABC	24	+		
2	ABC	48	0	27.53	<.01
	ABC	48	+		
3	DBC	24	0	.06	>.32
	DBC	24	+		
4	DBC	48	0	.42	>.32
	DBC	48	+		
5	ABC	24	0	3.09	≃.08
	DBC	24	0		
6	ABC	48	0	1.34	>.16
	DBC	48	0		
7	ABC	24	+	25.49	<.01
	DBC	24	+		
8	ABC	48	+	31.02	<.01
	DBC	48	+		

* 0 = No PVPN inj. + = PVPN inj.

toxin administration(14). In the present investigations PVPN* was tested for a possible protective effect against brucella endotoxin, the 2 agents being administered concomitantly.

Materials and methods. Mice, 5 to 6 weeks of age, were used for testing. The mice had Purina Fox Chow and tap water *ad libitum* and were kept in an airconditioned room, maintained at about 78°F, illuminated by day and darkened by night. A first series of experiments was carried out on hybrids of the A and C stocks (ABC mice). Because of availability, further experiments were carried out on DBC mice, obtained by mating DIF_1 hybrids to the D (dilute brown) stock. As the results on DBC mice were found to be at variance with those previously obtained on ABC mice, a third series of experiments was undertaken. The effects of endotoxin in each of these additional experiments with and without added PVPN were compared alternately in ABC and DBC mice. A Boivin-type of endotoxin made from a smooth culture of *Brucella melitensis* was employed, the details for its preparation having been presented elsewhere(3). Different lots of endotoxin were employed, the doses averaging 1.5 mg per 20 g of body weight. But in any given experiment the same lot of endotoxin was used. A 6% solution of PVPN was used in a dose of 0.5 ml per mouse. All injections were made into the tail vein. Except for one experiment on ABC mice in which it was given 5 minutes after the endotoxin, the PVPN was introduced simultaneously through the same syringe containing the endotoxin. Mortality rates are reported for 24 and 48 hours post-injection. The rates at these times were representative of the PVPN effect recorded in some experiments at shorter and longer intervals as well.

Results. The results of all the experiments are summarized in Fig. 1, and a statistical analysis of these data is shown in Table I. As noted in Fig. 1, a total of 453 ABC mice and 350 DBC mice were used in these studies. The outstanding and consistent result was that ABC mice given endotoxin and PVPN survived in significantly larger numbers than DBC animals treated in the same manner. While PVPN protected ABC mice against the lethal action of the endotoxin, this protection

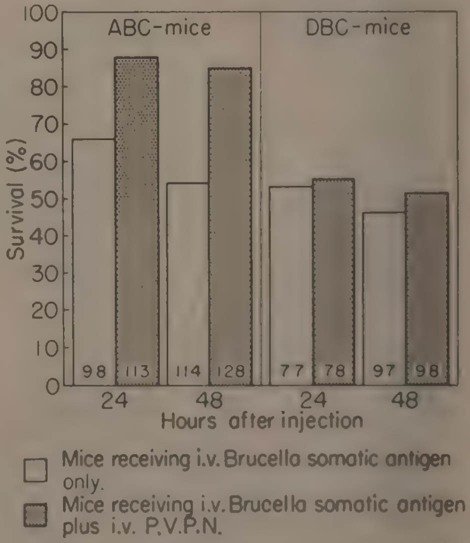

FIG. 1. Comparative survival rates in populations of ABC mice and DBC mice given brucella endotoxin alone, and brucella endotoxin plus Periston N (PVPN). No. of mice constituting each treatment group is shown at bottom of corresponding column.

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* Obtained from Die Farbenwerke Bayer, Leverkusen, Germany.

was lacking in the DBC mice, (Fig. 1) from the rates recorded at 24 as well as at 48 hours after injection of endotoxin. When the number of mice comprising these comparative studies is taken into consideration, the differences in per cent of survivals are quite significant. That PVPN protected ABC mice against endotoxin is supported by the results of the statistical analyses given in comparisons 1 and 2 of Table I.

The data in Fig. 1 also suggest that ABC mice may be less susceptible to the endotoxin than the DBC mice, and this might account for the protection offered to ABC mice by PVPN. The differences in survival rates between ABC and DBC mice at 24 and 48 hours after injection are however slight, and they are not significant at the 5% level, as seen in comparisons 5 and 6 in Table I. This slight difference in the susceptibility to the endotoxin does not appear to be sufficient to explain the large difference between ABC and DBC in their response to PVPN. The latter difference is analyzed in comparisons 7 and 8 in Table I, based on survival rates in those groups of ABC and DBC given PVPN as well as endotoxin. Whether data for survival rates at 24 or 48 hours are analyzed, the inter-population differences in survival rates are significant below 1% ($P<0.0005$).

Discussion. These experiments demonstrate that PVPN provides protection for mice against Brucella endotoxin. PVPN significantly increased survival rates of ABC mice when given 5 minutes after endotoxin injection, as well as when given in the same syringe with the endotoxin. But this protection is limited, with populations of ABC mice protected, but not DBC mice. Only a single injection of PVPN was given in evaluating the effect on survival rates. It would be of interest to see whether repeated injections of PVPN may have more general protective effects. In evaluating the effects of polymers in animals given endotoxin, two major considerations must be kept in mind. There exist not only intra-species differences in the response to the polymer, as the present experiments emphasize, but varying results must be anticipated when interpreting the differences between species of animals. When PVP alone is administered to dogs, shock is produced(16). Further differences within the same species may be forthcoming if there is a variation in the molecular weight of the polymer. Moreover, under certain experimental circumstances, related compounds of larger molecular weight have been found to be harmful in rabbits(17).

Summary and conclusions. Brucella endotoxin was administered intravenously to groups of mice and survival rates were recorded at 24 and 48 hours post-injection. The effect of a synthetic polymer on such survival rates was evaluated in 2 genetically different populations of mice—ABC mice (hybrids of the A and C stocks) and DBC mice (obtained by mating DIF_1 hybrids to the D stock). The polymer, polyvinylpyrrolidone (PVP) was used, in the form of Periston N (PVPN), having a molecular weight about 12,600. The mortality rates induced by endotoxin were modified favorably by PVPN in ABC mice, but not in DBC mice. It may be concluded that PVPN beneficially influences survival from Brucella endotoxin in at least one population of hybrid mice. The observation that another population of mice did not gain appreciable benefit from the same dose of PVPN may deserve further studies aimed at elucidating the mechanisms of host response to this agent.

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1. Spink, W. W., and Anderson, D., *J. Clin. Invest.*, 1954, v33, 540.

2. Weil, M. H., MacLean, L. D., Visscher, M. B., and Spink, W. W., *Trans. Assn. Am. Phys.*, in press.

3. Halberg, F., and Spink, W. W., *Lab. Invest.*, 1956, v5, 283.

4. Abernathy, R. S., and Halberg, F., *J. Lab. and Clin. Med.*, 1955, v46, 790.

5. Abernathy, R. S., and Spink, W. W., *J. Clin. Invest.*, in press.

6. Halberg, F., Spink, W. W., and Bittner, J. J., *Endocrinology*, 1956, v59, 380.

7. Halberg, F., and Spink, W. W., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 222.

8. Hecht, G., and Weese, H., *Münch. med. Wochensch.*, 1943, v90, 11.

9. Bennhold, H., and Schubert, R., *Z. f. exp. Med.*, 1943, v113, 722.

10. Bennhold, H., *Deutsche med. Wochensch.*, 1951, v76, 1485.

11. Schubert, R., *ibid.*, 1951, v76, 1487; 1954, v79, 170.

12. Dieckoff, J., *Die Therapiewoche*, 1952, v16, 1.

13. Bickel, E., and Dieckhoff, J., *Z. f. die ges. Inn. Med. u. Grenzgeb.*, 1955, v3, 116.

14. Krech, U., *Z. f. Immunit.*, 1952, v109, 177.

15. Dave, B. T., Borkar, S. A., Joag, G. G., Lewis, R. A., and Patel, J. C., *Exp. Med. Surg.*, 1955, v13, 293.

16. Halpern, B. N., *Ciba Foundation Symposium on Histamine*, Little Brown and Co., Boston, 1956, p92.

17. Brunson, J. G., *Arch. Path.*, 1955, v60, 593.

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Fibrinolytic and Coagulant Activities of Certain Snake Venoms and Proteases.* (22647)

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In a search for an ideal agent for the intravascular dissolution of thrombi and emboli in man, investigators have studied heparin(1,2, 3), coumarins(4), trypsin(5), chymotrypsin (3), streptokinase(6), staphylokinase(7), and plasmin(8). Certain snake venoms have long been known to be fibrinolytic *in vitro*. In addition, many of these are also known to contain other proteins with coagulant, hemolytic, neurotoxic or other properties which would contraindicate their clinical use. A survey of available snake venoms was undertaken to compare their *in vitro* lytic actions against fibrinogen, fibrin, formed fibrin and formed whole blood clots with various other proteolytic agents. Coagulant and hemolytic properties were also studied.

Materials. Bovine fibrinogen was a 1% solution of Armour's Fraction I. Human fibrinogen was a crude fraction prepared by 25% saturation of human plasma with ammonium sulfate, collection of the precipitate, dialysis, and adjustment of the clottable protein concentration to 0.5%. Plasmin was a spontaneously activated dog serum unglobulin fraction(9). Plasminogen was prepared from human barium sulfate adsorbed plasma by 20-fold dilution, adjustment of pH to 5.3, collection of precipitate and dissolution in a volume of 0.85% NaCl equal to 1/10 the original plasma volume. The fibrinogen was removed as fibrin after addition of a trace of thrombin (1 unit/10 ml). Streptokinase (Varidase, supplied through the courtesy of Lederle Laboratories) was dissolved in 0.85% NaCl to contain 200 units per ml. Uroprotease was prepared from human urine as described by Celander and Guest(10). Trypsin and chymotrypsin (crystalline, courtesy Armour Laboratories) were 0.1% solutions in 0.85% NaCl. Ficin (Delta), bromelain (courtesy Pineapple Research Institute of Hawaii), papain (Worthington), and pepsin (Worthington) were prepared as 1% solutions in 0.85% NaCl. Snake venoms (supplied through the courtesy of Wyeth and Co.; Ross Allen's Reptile Institute; Burroughs, Wellcome; and Hynson, Wescott and Dunning, Inc.) were dissolved as 1% solutions in 0.85% NaCl. *Stypven* (Burroughs, Wellcome) was used as a 0.2% solution. Thrombin (Thrombin, Topical, Parke Davis) was prepared to contain 100 units per ml.

Methods. Fibrinolysis was estimated by observing lysis time in a clot containing 0.2 ml material to be tested, 0.2 ml fibrinogen, 0.2 ml borate buffer (pH 7.6) and 0.05 ml thrombin. Intimate mixing of the lytic agent and fibrin was obtained by a 2-tube mixing method(11), in which the fibrinogen and buffer were poured into the lytic agent and thrombin, at zero time, and subsequently poured back and forth three times. ***Formed clot lysis.*** ***Fibrin***: Human fibrinogen clots

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† Post-doctorate Fellow, Life Insurance Medical Research Fund.

were prepared by adding 0.05 ml thrombin to 0.2 ml fibrinogen, mixing and allowing the clot to stand 15 min. The clot was freed from the tube by a sharp rap and suspended in 0.2 ml material plus 0.2 ml buffer. Lysis time was measured from time of addition of material to time of complete disappearance of the clot. *Whole blood*: 0.2 ml of fresh human blood was pipetted into each of a series of tubes, clotting and retraction allowed to proceed for 1 hour at 37°C and 0.2 ml of the test material added. *Fibrinogenolysis* was tested by incubating 1.0 ml material with 1.0 ml human fibrinogen and testing at intervals for residual fibrinogen by adding 0.2 ml aliquots to 0.05 ml thrombin. The time at which no clot formed was taken as the fibrinogenolysis time.

Thrombic activity was measured by determining the clotting time after addition of 0.1 ml material to 0.1 ml human fibrinogen. *Thromboplastic* activity was determined, in materials which were not thrombic, by substituting 0.1 ml material for thromboplastin in a prothrombin time test. *Hemolytic* and *hemagglutinating* activities were observed in tubes containing 0.2 ml material and 0.2 ml 1% dilution of human whole blood. All procedures were carried out at 37°C.

Results. Actual lysis times observed in the various fibrinolytic systems are shown in Table I. Venoms of the Crotalus, Bothrops and Agkistrodon groups were fibrinolytic, while those of the Cobras (crude Cobra, *N. naja, N. flava*) were not. *V. russellii* was fibrinolytic but inactive against the formed clots. *Stypven*, made from *V. russellii*, was inactive. All of the other agents were fibrinolytic except pepsin, plasminogen alone, and streptokinase in the bovine system. Ficin, bromelain and papain were the only ones with significant activity in the whole blood clots. When trypsin and chymotrypsin were tested as 1% rather than 0.1% solutions, no clots were formed in the fibrinolytic systems and the whole blood clots were lysed in 1 to 2 hours.

TABLE I. Fibrinolytic Activities.

	Fibrinolysis (min.)		Clot lysis (min.)	
	Bovine fibrin	Human fibrin	Human fibrin	Human blood
C. basiliscus	<1	5	48	180
C. atrox	3	5	90	62
C. horridus	5	19	151	480
C. adamanteus	15	21	280	480
C. hellerii	NC	8	480	480
C. terrificus	69	>120	1080	>1080
B. neuweidii	4	3	48	68
B. neuweidii 0.1%				>1080
B. jararaca	5	4	65	66
B. atrox	7	7	90	180
B. alternatus	20	21	155	480
A. piscivorus	4	8	65	65
A. mokasen	6	7	65	180
Crude Cobra	>120	>120	>1080	>1080
N. naja	>120	>120	>1080	>1080
N. flava	>120	>120	>1080	>1080
V. russellii	<1	5	>1080	>1080
'Stypven' 0.2%	>120	>120	>1080	>1080
Plasmin (dog)	4	5	148	>1080
Plasminogen (human)	>120	>120	>1080	>1080
Streptokinase (SK)	>120	5	142	>1080
Uroprotease (UP)	8	10	92	>1080
Plasminogen + SK	2	2	57	>1080
Plasminogen + UP	12	9	84	>1080
Trypsin 1 %	<1	<1	25	72
" .1%	<1	<1	28	>1080
Chymotrypsin 1 %	1	1	25	120
" .1%	1	2	32	1080
Ficin	5	31	1080	240
Bromelain	<1	2	280	540
Papain	2	2	900	160
Pepsin	>120	>120	>1080	>1080
Saline	>120	>120	>1080	>1080

Inability of some of the preparations to lyse whole blood clots might depend upon the sensitivity of the enzymes to the serum antiprotease present in the whole blood system. Therefore, additional tests were performed by incubating the various agents with human serum and measuring residual lytic activity. The active snake venoms, ficin, bromelain and papain were inhibited far less than plasmin, uroprotease, and streptokinase-plasminogen. Trypsin and chymotrypsin showed variable activity: either increased or decreased, depending upon time of incubation and strengths of serum and enzyme, suggesting that two processes were occurring simultaneously: inhibition and activation of the serum plasminogen.

Thrombic activity, *i.e.* ability to gel fibrinogen, was found in 8 venoms and papain (Table II). Only trypsin, *V. russellii*, and

TABLE II. Toxic Activities.

Agent	Thrombic (sec.)	Thromboplastic (sec.)	Fibrinogenolytic (min.)	Hemolytic	Hemagglutinating
C. basiliscus	>600	>600	5	0	0
C. atrox	>600	>600	5	0	+
C. horridus	21.0	—	—	0	0
C. adamanteus	10.0	—	—	0	0
C. hellerii	45.0	—	—	0	0
C. terrificus	16.6	—	—	0	0
B. neuweidii	10.8	—	—	0	+
B. jararaca	18.4	—	—	0	0
B. atrox	11	—	—	0	0
B. alternatus	21.4	—	—	0	0
A. piscivorus	>600	>600	15	0	+
A. mokasen	>600	>600	10	0	+
Crude cobra	>600	>600	10	+	0
N. naja	>600	>600	10	+	0
N. flava	>600	>600	5	+	0
V. russellii	>600	30	25	0	0
"Stypven"	>600	19.0	>60	0	0
Plasmin (dog)	>600	100	>60	0	0
Plasminogen (human)	>600	56.5	>60	0	0
Streptokinase (SK)	>600	100	26	0	0
Uroprotease (UP)	>600	100	>60	0	0
Plasminogen + SK	>600	100	25	0	0
" + UP	>600	100	60	0	0
Trypsin .1%	>600	12.8	<¼	0	0
Chymotrypsin .1%	>600	68	<¼	0	0
Ficin	>600	63	¾	+	0
Bromelain	>600	>600	15	+	0
Papain	25.0	—	—	0	0
Pepsin	>600	100	>60	+	0
.85% NaCl	>600	95	>60	0	0

its derivative *Stypven* were thromboplastic. Certain snake venoms appeared anti-coagulant, *i.e.* thromboplastin time longer than that of saline. In each case this appeared due to fibrinogenolysis.

All of the snake venoms that could be tested (those which were not thrombic) were fibrinogenolytic, as were the other fibrinolytic enzymes. The cobra venoms were remarkable in being fibrinogenolytic but not fibrinolytic. Hemolysis was produced by the cobra venoms, ficin, bromelain and pepsin. Agglutination of human red cells, seen grossly and confirmed microscopically, was caused by *C. atrox, B. neuweidii, A. piscivorus* and *A. mokasen*.

The possibility that strongly fibrinogenolytic venoms showing no thrombic activity might actually be thrombic as well at a lower concentration was investigated. Fibrinogen was incubated with various strengths of three such venoms (*A. mokasen, A. piscivorus, C. basiliscus*) from 1% down to .00002%, the latter figure being far below the concentration necessary for fibrinogenolysis to be evident. At none of these strengths was thrombic activity apparent.

Discussion. Fontana, in 1787(12), noted the blood to remain fluid in animals dead of viper bite. Since then various venoms have been found to exhibit various activities on the coagulation and fibrinolytic mechanisms(13, 14,15). Therapeutic use in hemorrhagic diseases has not been encouraging. Other properties of venoms, including hemolysins(16), neurotoxins(17) and hyaluronidase(18) have been the subject of many investigations. The data here presented indicate that a number of snake venoms actively lyse human blood clots and appear to have a certain theoretical advantage over the more common fibrinolytic agents in that they are less inhibited by human serum antiprotease. However, these venoms are all either fibrinogenolytic, thrombic, thromboplastic, hemolytic or hemagglutinating, thus contraindicating their clinical use in crude form. Fractionation of whole venoms has been reported(19,20) and it seems conceivable that a pure fibrinolytic fraction, devoid of toxic activities, might be separated and prove of therapeutic value.

Summary. Of the 16 snake venoms studied, 11 actively lysed human blood clots. However, only one of these, *C. basiliscus,* was devoid of thrombic, hemolytic, and hemagglutinating properties. This venom was fibrinogenolytic as well as fibrinolytic. The possible therapeutic use of certain venoms as dissolving agents for intravascular clots presents a theoretical advantage over most other fibrinolytic agents in that their fibrinolytic activity is not readily inhibited by human serum. Fractionation of a pure fibrinolytic principle may be possible.

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1. Loewe, L., and Hirsch, E., *J. Am. Med. Assn.*, 1947, v133, 1263.
2. Rabinovitch, J., and Pines, B., *Surgery,* 1943, v14, 669.
3. Didisheim, P., *Bull. Johns Hopkins Hosp.,* 1954, v94, 351.
4. Wright, H. P., Kubik, M. M., and Hayden, M., *Brit. Med. J.,* 1953, v1, 1021.
5. Innerfield, I., Schwarz, A., and Angrist, A., *J. Clin. Invest.,* 1952, v31, 1049.
6. Tillett, W. S., Johnson, A. J., McCarty, W. R., *ibid.,* 1955, v34, 169.
7. Lewis, J. H., and Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 677.
8. Cliffton, E. E., Grossi, C. E., and Cannamela, D., *Ann. Surgery,* 1954, v139, 52.
9. Lewis, J. H., and Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 184.
10. Celander, D. R., and Guest, M. M., *Fed. Proc.,* 1955, v14, 26.
11. Lewis, J. H., and Ferguson, J. H., *Am. J. Physiol.,* 1952, v170, 636.
12. Fontana, on Poisons. Translated by J. Skinner, London, 1787.
13. Noc, F., *Ann. Inst. Pasteur,* 1904, v18, 387.
14. Eagle, H., *J. Exp. Med.,* 1937, v65, 613.
15. Essex, H. E., *Physiol. Rev.,* 1945, v25, 148.
16. Kellaway, C. H., *Bull. Johns Hopkins Hosp.,* 1937, v60, 1.
17. Zeller, E. A., *Adv. Enzymology,* 1948, v8, 459.
18. Duran-Reynals, F., PROC. SOC. EXP. BIOL. AND MED., 1938, v38, 763.
19. Rosenfeld, S., and Rubenstein, J., *J. Lab. and Clin. Med.,* 1941, v27, 45.
20. Polson, A., Joubert, F. J., Haig, D. A., *Biochem. J.,* 1946, v40, 265.

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Effect of Thyroxine on Liver Tyrosine-glutamic Acid Transaminase. (22648)

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In the course of studying the effects of different hormonal states and hormone administration upon activity of several rat liver enzymes it was observed that dietary treatment with 1% thyroid for 8 days or more resulted in marked inhibition of liver tyrosine oxidation(1). Furthermore, the degree of inhibition was equally significant in normal animals and in animals under conditions of varying hormonal state, whereas certain of the other enzymes were affected by thyroid only under restricted conditions. This suggested a direct effect of the hormone* upon the tyrosine oxidase system. Since low dietary protein decreased activity of homogenate preparations (1) and inanition has been reported to enhance tyrosine oxidation in soluble preparations(2) this effect was further investigated using *in vitro* technics.

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* Reference to thyroxine hormone means thyroxine itself or whatever active form to which it may be converted.

Methods and materials. Mature Long-Evans strain male rats weighing between 200 and 400 g were used as a source of liver tissue. The enzyme was prepared by homogenizing fresh, chilled liver with 2 volumes of cold 0.14 M KCl and centrifuging at 30,000 rpm for 45 minutes in a refrigerated Spinco centrifuge with a No. 40 rotor. The clear supernatant was used as the enzyme preparation. Enzyme activity was measured by use of an automatic recording Beckman spectrophotometer with a thermostat-controlled heating chamber at 37°C. Tracings from the recorder measured changes in optical density at 320 mμ (U. V. Light) which measured appearance of the enol form of p-hydroxyphenyl pyruvate (HPP) and tautomerization of the keto form to the enol form of HPP by tautomerase(3). Keto HPP shows end absorption in U.V. light while the enol form absorbs maximally at 300 mμ. The transaminase-tautomerase system included: 6 μM tyrosine in phosphate buffer(3), 0.1 ml of 0.1 M α-keto glutarate at pH 7.0, 1 ml of 0.2 M phosphate buffer at pH 7.0, 0.3 ml of soluble enzyme preparation (pH 7.0) and water to make a total volume of 3.6 ml. The final pH of the system was 7.0. The system used to measure tautomerase activity included: 6 μM keto HPP, pH 6.8, 1.0 ml 0.2 M phosphate buffer at pH 7.0, 0.3 ml enzyme and water to make 3.6 ml. L-thyroxine was dissolved in 0.02 N NaOH (pH 8)(4) and was added to the system in 0.1 ml and pre-incubated with the enzyme for 2 minutes at 25°C before addition of substrate, however, this procedure was not necessary to obtain the hormone action. The reaction was carried out in 1 cm quartz cells and the complete system was set to zero optical density immediately after the addition of the substrate. Either tautomerase or transaminase-tautomerase activity has been expressed as the change in optical density at 320 mμ in 10 min. (Δ O.D. 320 mμ/10 min.). One ml of the enzyme contained approximately 1.8 mg Kjeldahl nitrogen.

Results. When the fresh enzyme preparation was used to measure enol HPP accumulation with tyrosine as substrate, equilibrium enol HPP formation was reached in about 10 min. Further oxidation of HPP by oxidative enzymes could be seen in 20 minutes when enol HPP had nearly disappeared. In the presence of 10^{-4} M thyroxine the overall reaction rate was reduced by about 60%. Since Knox and Knox(6) have demonstrated that the transaminase reaction limited the overall rate of the system converting tyrosine to fumarate and acetoacetate and from kinetic studies of this system measuring QO_2(1) it appeared that the site of inhibition by thyroxine must be early in the tyrosine metabolizing sequence. After 48 hours of aging the enzyme at 5°C, further oxidation of enol HPP could no longer be detected during 60 minutes of reaction with tyrosine and α-ketoglutarate in the system as substrates. This meant that the residual activities were those of transaminase (E_1) and tautomerase (E_2) whose activity had not decreased significantly during this time:

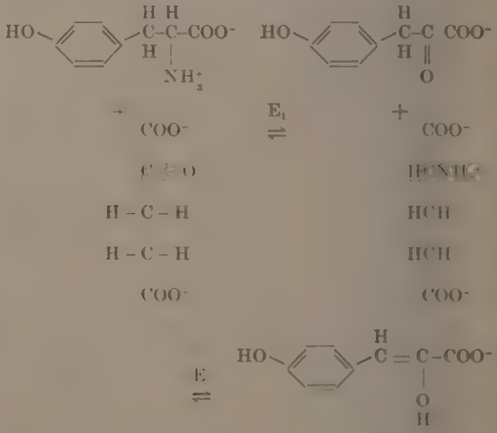

Table I shows the effect of increasing concentrations of thyroxine on coupled transaminase-

TABLE I. Effect of Increasing Concentrations of Thyroxine upon Tyrosine Glutamic Acid Transaminase-Tautomerase Activity.

Enzyme* concentration (mg N)	L-Thyroxine concentration (M)	Transaminase-tautomerase activity (△ O.D. 320 mμ/10 min.)
.54		.274
"	5.10	.121
"	1.10	.077
"	2.10	.050

* 3 day aged enzyme.

TABLE II. Effect of Thyroxine upon Activities of Coupled Transaminase-Tautomerase and Tautomerase.

Thyroxine conc. (M)	Substrate (6 μM)	Enzyme activity* (Δ O.D. 320 mμ/10 min.)	% inhibition
None	L-tyrosine	.220	—
10^{-4}	"	.082	63
None	keto HPP	.330	—
10^{-4}	"	.300	9

* 3 day aged enzyme.

tautomerase activity. Since the results showed significant effects upon this system it was essential to determine which enzyme was being inhibited by the hormone.

Table II shows the effect of added thyroxine to the coupled enzyme system and also upon the tautomerase reaction which could be isolated from E_1 by omitting α-ketoglutarate from the system and supplying keto HPP as substrate. The formation of enol HPP was then measured in the aged preparation which was free of detectable oxidative steps during the experimental period. While only a small inhibition of E_2 activity was noted in the presence of thyroxine a large inhibition occurred in the E_1E_2 reaction which indicated that the site of thyroxine action occurred at the transaminase level. In addition, this inhibition appeared to be non-competitive in nature as determined by the method of Ackermann and Potter(5).

Discussion. The possibility that thyroxine may act as an inhibitor of other transaminating enzymes is being investigated. The effective levels of thyroxine indicate that this reaction may be of physiological importance. *In vivo* data(1) on tyrosine oxidation add credence to this point. The significance of such a mechanism in regard to protein synthesis is obvious. Previous work with diiodotyrosine(1) indicates that this substance inhibits at the substrate level (competitive inhibition) while thyroxine is more effective at lower concentrations and appears to be binding the enzyme (non-competitive inhibition) perhaps analogous to thyroglobulin formation. Binding of thyroxine has been observed to occur in serum by a "thyroxine binding protein"(7) presumably of the G_1 globulin fraction. A report of *in vivo* studies and mechanism of inhibition will appear later.

Summary. Thyroxine in concentrations as low as 5.10^{-5}M has been shown to markedly inhibit transaminase-tautomerase activity of the liver tyrosine oxidase system. The transaminase enzyme appears to be the most directly involved in the inhibition as determined by difference studies.

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1. Litwack, G., to be published.

2. Kring, J. P., and Williams, J. N., Jr., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 97.

3. Knox, W. E., in *Methods in Enzymology*, Acad. Press, N. Y., 1955, v2, 287.

4. Hoch, F. L., and Lipmann, F., *Proc. National Acad. Sci.*, 1954, v40, 909.

5. Ackermann, W. W., and Potter, V. R., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 1.

6. Knox, W. E., and Le May-Knox, M., *Biochem. J.*, 1951, v49, 686.

7. Robbins, J., and Rall, J. E., *J. Clin. Invest.*, 1955, v34, 1324.

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Growth and Regeneration of *Schistosoma mansoni in vitro*.*† (22649)

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The development of technics for the cultivation of the parasitic trematodes of man has progressed slowly. Studies on the most important human pathogens in this group, the schistosomes, have dealt primarily with the longevity of adult worms under artificial conditions in media containing serum and with attempts to define the constituents of serum essential for their survival. *Schistosoma japonicum* has survived *in vitro* for as long as 82 days in a rabbit serum medium(1), and for almost 5 months in horse serum-Ringer's solution containing washed red cells(2). Mature *S. mansoni* have been maintained for 18 days in ox serum(3), and for 2 months in horse serum(4). Egg production has been reported in a human serum system(5). With a chemically defined medium, a survival time for *S. mansoni* of 5 days has been noted(6).

Smyth(7) has emphasized that in studies on helminths *in vitro* criteria for demonstration of "growth" as contrasted to mere "survival" need to be developed. In this paper we will present evidence that growth of immature schistosomes maintained *in vitro* occurred, as manifested by an increase in size and by regeneration of amputated portions of certain of the worms.

Materials and methods. Specimens of immature *S. mansoni* were obtained employing aseptic technics from the portal system of white mice infected percutaneously in our laboratory. In addition, schistosomulae were recovered from peritoneal washings and from washings of liver fragments of mice inoculated with cercariae by the intraperitoneal route; these infected mice were supplied through the courtesy of Dr. Henry van der Schalie of the University of Michigan.

Infected mice were lightly etherized and then killed by cervical fracture. The peritoneal cavity was flushed with culture fluid (CF) in the case of mice infected by inoculation. The portal vessels and liver were then removed to separate Petri dishes containing CF and worms recovered by the teasing apart of the tissues under a dissecting microscope. Mice that had been infected by the percutaneous route were given 0.4 ml of heparin sodium (Liquaemin, Organon) intraperitoneally 5 minutes before being sacrificed.

The worms were transferred with Pasteur pipettes through several changes of fluid and then placed singly or in pairs in rubber-stoppered or screw-capped test tubes (15 x 150 mm) containing 1.4 ml of CF. The culture fluid was that used by us for maintenance of human cells *in vitro*, with the single omission of soybean trypsin inhibitor. It consisted of 45% bovine amniotic fluid(8), 45% Hanks' balanced salt solution(9), 5% inactivated horse serum, and 5% beef embryo extract, with penicillin, streptomycin and phenol red as described(8). Whenever necessary, the pH was adjusted to about 7.4 prior to use by gassing with 5% CO_2 in air. Cultures were maintained in an upright position at 37°C. At 2- to 4-day intervals, the CF was withdrawn and fresh medium substituted. The basic medium was supplemented in certain experiments by the addition to each tube at weekly intervals of fresh washed mouse red blood cells. After each addition, the erythrocytes were left in the cultures for 2 to 4 days and then removed. Cells for this purpose were obtained from young female white mice; such mice were heparinized, sacrificed as described above, and bled from the exposed heart. The cells were washed and centrifuged 3 times in CF. Approximately 0.3 ml of packed cells was resuspended in 2.0 ml of medium and 2 drops of the resulting suspension added to each culture. Final concentration

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* This work was supported in part by a research grant (E-1023) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Public Health Service, and in part by a grant from the United Fruit Co.

† We are indebted to Mr. John Carabitses for the preparation of the photomicrographs.

of red cells in the culture medium was determined to be equivalent to a hematocrit of 3 to 5.

All subsequent manipulations of the cultures, with exception of photographic procedures, were carried out in a warm room at 32°C. The schistosomes were observed daily under a compound or dissecting microscope. This procedure was facilitated by gently rotating the culture in an almost horizontal position until the worm adhered to the tube wall out of the fluid. Serial camera lucida drawings were made in order to document changes in size. Inasmuch as constant contraction and extension of the worms was occurring, exact determination of length was difficult. Therefore, at each observation period 3 to 6 outline drawings were made per worm. A "map measurer" calibrated by mensuration of the projected image of a slide micrometer, was run along the axis of each outline. Length was arbitrarily derived by averaging sets of measurements. Serial drawings of worms less than 0.5 mm could not be obtained, since worms of this size could not regularly be induced to adhere to the wall of the culture tube. In a number of instances, interval photographs were taken. Tracings of these photographs were transferred to graph paper for calculation of total area as a measurement of body growth. Results generally corroborated data on length as obtained by multiple camera lucida drawings.

The CF, removed from each tube at the first change of medium, was cultured in thioglycollate broth and on Sabouraud's agar to reveal bacterial or fungal contaminants. Similar control cultures were repeated at intervals. No bacterial contamination was encountered, but growth of a mold resulted in loss of 3 worms.

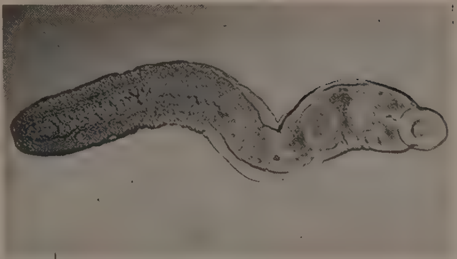

FIG. 1. Male schistosome (#905) recovered from mouse on 16th day of infection and photographed on 11th day *in vitro*. Length approx. 2 mm.

Results. A total of 78 schistosomes was studied *in vitro* for various periods of time. Although all worms showed some increase in length, irrespective of initial size, the survival period *in vitro* of worms measuring less than 0.5 mm was limited. The results of experiments with schistosomulae recovered from the peritoneal cavity of mice 6 to 10 days after injection of cercariae are summarized in Table I. In this series, erythrocytes were not added to the medium. A similar experiment was initiated using 11 worms obtained from mice on the 16th day of infection. For 2 weeks these were maintained in individual cultures on CF alone; at that point 5 of the worms were given red cells which appeared to increase activity and prolong slightly their survival. Two worms were alive after 35 days in culture, at which time the experiment was terminated; one, a female (#911) had increased in length from 0.98 mm to 4.58 mm, while the other, a male (#905) had increased from 1.52 to 4.08 mm. These schistosomes are pictured in Fig. 1 and 2. The results of serial measurements on the 16-day-old schistosomulae are summarized in Table II.

Immature schistosomes, derived from mice 30 or more days after percutaneous infection,

TABLE I. Survival of Schistosomulae *In Vitro*.

No. of worms in group	Time in days between infection of mice and recovery of worms	Original length (mm)*	Final length (mm)*	Avg days survival *in vitro*
6	6	.12–.25	.25– .50	3.3
6	9	.15	.50– .75	10.5
4	10	.25	.75–1.2	10.7

* Length estimated; serial measurements not done.

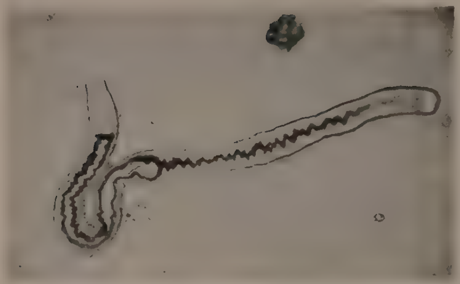

FIG. 2. Female schistosome (#911) recovered from mouse on 16th day of infection; photographed on 24th day *in vitro* and 24 hr after red cells had been added to the medium. Length 2.4 mm.

and having an initial length exceeding 1.5 mm, generally survived for many weeks on CF supplemented with red cells. Thus, 40 trematodes in this category remained alive and displayed vigorous feeding activity for periods as long as 87 days. Serial measurements on 3 male worms and 1 female in this group are presented graphically in Fig. 3. Accelerated somatic movement and peristaltic activity usually followed ingestion of the added red cells. After an exposure of 8 to 24 hours to the erythrocytes, ceca previously almost devoid of pigment would become distended with black material; usually such pigment disappeared within 48 hours following the withdrawal of red cells from the system.

Three pairs of larger worms (3-5 mm), each pair consisting of a male and a female, were placed in separate tubes. All began to copulate *in vitro*. In one case this attitude was maintained for 2 months, while the other pairs parted and rejoined at irregular intervals. No eggs were produced.

No systematic study was undertaken of the ability of schistosomes to regenerate amputated portions, but observations were made on a limited number of worms accidentally traumatized during recovery procedures. Four worms that lacked the posterior ⅓ to ½ of the body were noted to regenerate the missing part *in vitro* within 10 to 20 days. Ceca, cut just proximal to the point of union, were seen to unite and form the usual single posterior trunk during the regenerative period. In one specimen, a large cyst-like bladder developed in the regenerating posterior third. Although the excretory system remained imperfect, this individual survived during the 75-day observation period. Two schistosomes lacking the anterior third of the body were observed for 23 days, during which time there was no noticeable diminution of activity.

TABLE II. Survival and Growth *In Vitro* of 16-Day Schistosomulae.

Worm No.	Original length (mm)*	Final length (mm)*	% size increase	No. days survival
901	1.0	1.23	23	21
902	.94	1.01	7	26
904	.46	.61	33	25
905	1.52	4.08	168	(35)†
906	.62	.92	48	23
908	.67	1.09	63	25
909	.63	.87	38	17
910	.54	.67	24	17
911	.98	4.58	367	(35)†
912	.41	.61	49	17
913	.58	.61	5	17

* Averages derived from camera lucida drawings.

† ( ) = alive at end of experiment.

Discussion. The results of these preliminary studies indicate that a nutrient medium suitable for the cultivation of mammalian cells will also induce growth of *S. mansoni in vitro* as evidenced by an increase in length and by the regeneration of somatic amputations. That this nutrient system is not opti-

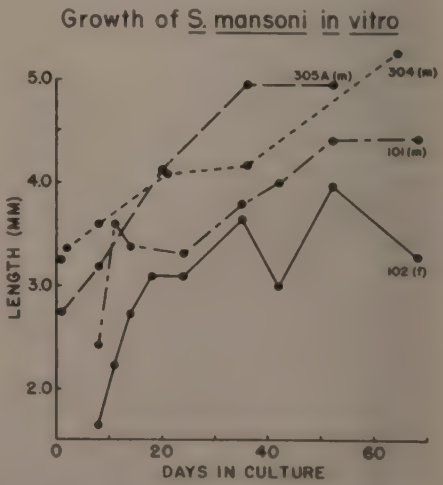

FIG. 3. Serial measurements derived from camera lucida drawings of 4 schistosomes maintained *in vitro* on CF supplemented with red cells.

mal is clearly indicated by the limited survival periods of the small specimens. The apparent stimulatory effect of ingested red cells on the larger worms requires further investigation. It is to be noted that Moore and Meleney(10) have suggested that the defective maturation of *S. mansoni* in the peritoneal cavity of the mouse may be a reflection of the absence of blood upon which to feed. Although not yet ideal, the *in vitro* system here described offers a convenient means for the direct and prolonged observation of feeding schistosomes. The technic should, therefore, prove useful for a variety of experimental purposes, as well as offering a baseline for the development of improved media in the future.

Summary. Preliminary investigations on the growth of *Schistosoma mansoni in vitro* are reported. In a nutrient medium composed of bovine amniotic fluid, beef embryo extract, and horse serum, schistosomulae generally showed evidence of growth. The duration of survival was directly related to initial size. Worms that initially approximated 1.0 mm or greater in length were found to survive many weeks *in vitro;* the survival period of smaller schistosomulae was of much shorter duration. The addition of red cell suspensions to the basic medium was followed by active feeding and accelerated somatic movements. Growth of the schistosomes *in vitro* was manifested by increases in length of male worms to a maximum of 168%, and of female worms to 367%, and also by the observed regeneration of amputated portions of certain of the worms.

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1. Lee, C. U., and Chu, H. J., PROC. SOC. EXP. BIOL. AND MED., 1935, v32, 1379.
2. Chu, H. J., *China Med. J.*, 1938, Suppl. 2, 411.
3. Ross, O. A., and Bueding, E., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 179.
4. Newsome, J., and Robinson, D. L. H., *Ann. Trop. Med. and Parasit.*, 1954, v48, 194.
5. Robinson, D. L., *Trans. Royal Soc. Trop. Med, and Hyg.*, 1954, v48, 283.
6. Bueding, E., and Ruppender, H., cited in *Ann. Rev. Microbiol.*, 1953, v7, 307.
7. Smyth, J. D., *Exp. Parasit.*, 1956, v5, 260.
8. Enders, J. F., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 100.
9. Weller, T. H., Enders, J. F., Robbins, F. C., and Stoddard, M. B., *J. Immunol.*, 1952, v69, 645.
10. Moore, D. V., and Meleney, H. E., *J. Parasit*, 1955, v41, 235.

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Action of Thyroid Hormones on Brain Metabolism of Newborn Rats. (22650)

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Investigations have shown that the metabolism of the brain is better protected against interference, especially of the thyroid hormone, than other tissues(1a). It was reported(1) that thyrotrophic hormone can increase the brain oxidation of hypophysectomised rats, although it can seldom influence that of normal adult rats. It seemed that after hypophysectomy a regulating mechanism, which is responsible for the constancy of the brain metabolism, is disturbed. During the last few years it seemed promising to continue these experiments on animals whose anterior pituitary function was not yet fully developed. The brain of newborn rats seemed also to be particularly interesting owing to the extraordinary resistance of these animals to anoxaemia which enables them to survive in a carbon monoxide atmosphere(2).

Contrary to previous experiences with adult animals these investigations showed some significant changes after treatment with thyroid hormones.

Methods. Litters of Wistar rats were 5 to 11 days old at investigation, and weighed 8 to 20 g each. Each litter was divided equally, TABLE I. O_2 Consumption of Cerebral Cortex of Newborn Rats after Thyroxine Treatment.

		Controls (saline treated)		Thyroxine treated animals				
					Thyroxine treatment			
Litter No.	Age in days	No. of animals	Avg O_2 consumption/hr/100 mg fresh tissue, $mm^3 \pm S.D.$	No. of animals	Total dose, mg	No. of single daily doses	Avg O_2 consumption/hr/100 mg fresh tissue, $mm^3 \pm S.D.$	% differences as compared with controls
I	8	4	81.5 ± 5.5	4	.15	1	84.7 ± 7.5	+ 3.9
II	7	6	76.5 ± 7.0	6	.15	1	86.2 ± 5.8	+12.7*
III	8	4	84.0 ± 3.7	5	.15	2	93.6 ± 9.7	+11.4
IV	9	4	89.5 ± 5.7	4	.15	2	99.2 ± 1.7	+10.8*
V	8	4	78.0 ± 4.1	5	.15	3	91.2 ± 6.5	+16.9†
VI	8	6	97.2 ± 2.6	6	.25	3	109.8 ± 4.7	+13.0†
VII	9	5	92.2 ± 2.4	7	.25	5	102.1 ± 6.8	+10.7†
VIII	9	3	88.3 ± 2.6	3	.25	5	105.0 ± 1.7	+18.9†

* Significant at P 0.05. † Significant at P 0.01.

TABLE II. O_2 Consumption of Cerebral Cortex of Newborn Rats after Treatment with Thyroxine and Triiodothyronine.

		Treatment control animals (saline treated)		.1 mg triiodothyronine, daily for 4 days			.1 mg thyroxine, daily for 4 days		
Litter No.	Age in days	No. of animals	mm^3 O_2/hr/100 mg fresh tissue, mean ± S.D.	No. of animals	mm^3 O_2/hr/100 mg fresh tissue, mean ± S.D.	% differences as compared with controls	No. of animals	mm^3 O_2/hr/100 mg fresh tissue, mean ± S.D.	% differences as compared with controls
1	10	5	85.2 ± 6.3	4	96.8 ± 15.2	+13.5			
2	10	4	83.2 ± 4.9	4	110.0 ± 4.8	+32.1*			
3, 4, 5 comb.	10	7	104.7 ± 6.5	6	114.8 ± 3.1	+11.0*	7	115.4 ± 8.7	+11.0*
								Idem for 6 days	
6	10	2	99.5 ± 6.4	2	126.5 ± 9.2	+27.1	3	124.0 ± 5.6	+24.6†

* Significant at P 0.01. † Significant at P 0.05.

TABLE III. O_2 Consumption of Cerebral Cortex of Newborn Rats after Treatment with Thyrotrophic Hormone.

		Controls (saline treated)		Animals treated with thyrotrophic hormone				
					T.S.H. treatment			
Litter No.	Age in days	No. of animals	Avg O_2 consumption/hr/100 mg fresh tissue, $mm^3 \pm S.D.$	No. of animals	Total dose, i.u.	No. of single daily doses	Avg O_2 consumption/hr/100 mg fresh tissue, $mm^3 \pm S.D.$	% differences as compared with controls
I	8	3	92.7 ± 6.0	4	1.6	3	98.5 ± 10.1	+ 6.3
II	8	4	91.2 ± 4.8	5	1.6	3	96.8 ± 6.5	+ 6.1
III	8	4	90.7 ± 5.2	5	1.6	7	104.6 ± 4.6	+15.3*

* Significant at P 0.01.

TABLE IV. O_2 Consumption of Cerebellum of Newborn Rats after Thyroxine Treatment.

		Controls (saline treated)		Thyroxine treated animals				
					Thyroxine treatment			
Litter No.	Age in days	No. of animals	Avg O_2 consumption/hr/100 mg fresh tissue, $mm^3 \pm S.D.$	No. of animals	Total dose, mg	No. of single daily doses	Avg O_2 consumption/hr/100 mg fresh tissue, $mm^3 \pm S.D.$	% differences as compared with controls
I	8	5	133.4 ± 15.7	6	.25	5	145.8 ± 12.6	+ 9.3
II	8	4	127.0 ± 11.1	4	.25	5	145.0 ± 21.8	+14.2
III	11	4	121.7 ± 23.6	4	.15	3	151.5 ± 11.8	+24.5

TABLE V. % Dry Substance of Cerebral Cortex, Cerebellum and Medulla Oblongata of Newborn Rats Treated with Thyroxine, Triiodothyronine and Thyrotrophic Hormone.

Litter No.	Age in days	No. of animals	% dry substance, mean ± S.D. Cortex	Cerebellum	Medulla oblongata	No. of animals	% dry substance, mean ± S.D. Cortex	Cerebellum	Medulla oblongata	% difference as compared with controls Cortex	Cerebellum	Medulla oblongata
		Control animals, saline treated				.05 mg thyroxin daily, 3 days						
I	5	4	12.3 ± .25	12.7 ± .54	12.2 ± .27	4	12.8 ± .24	14.1 ± .27	12.9 ± .41	+5.9*	+11.5†	+ 5.6*
II	8	5	12.4 ± .25	13.2 ± .19	14.1 ± .32	4	13.2 ± .22	14.7 ± .20	14.9 ± .36	+6.4†	+11.0†	+ 5.8†
III	11	4	14.15± .31	14.3 ± .46	15.2 ± .36	4	14.7 ± .30	15.4 ± .38	16.8 ± .54	+3.5	+ 7.7†	+10.3†
						.1 mg tri-iodothyronine daily, 4 days						
IV	6	3	12.2 ± .12	13.9 ± .61	13.9 ± .73	4	13.2 ± .36	14.6 ± .22	14.3 ± .29	+8.7†	+ 4.9	+ 2.9
V	11	3	14.1 ± .33	15.1 ± .30	15.1 ± .29	3	15.0 ± .52	16.1 ± .21	16.3 ± .35	+6.1	+ 6.5†	+ 8.0†
						.2 i.u. thyrotrophic hormone daily, 7 days						
VI	10	5	13.6 ± .43	14.9 ± .20	15.1 ± .35	4	14.3 ± .15	15.7 ± .17	15.5 ± .24	+5.7†	+ 5.2†	+ 3.0
VII	10	6	13.2 ± .15	14.3 ± .29	14.6 ± .39	5	14.3 ± .49	15.1 ± .42	15.6 ± .25	+8.4†	+ 5.6†	+ 6.7†

* Significant at P 0.05. † Significant at P 0.01.

one half receiving daily injections of either thyroxine, thyrotrophic hormone, or triiodothyronine, and the other, or control half, receiving normal saline. The litters remained with the mother throughout the period of treatment. The animals were killed by decapitation, the brain quickly removed and, in order to expedite the start of the experiment, small tissue snippets were cut with a pair of fine eye scissors. These snippets were sufficiently thin to show the same oxygen consumption as brain slices, as had been established in a number of preliminary investigations. The snippets were weighed on a torsion balance with a capacity of 100 mg. Ninety to 95 mg of tissue snippets were investigated in conical Warburg vessels. Krebs Phosphate Ringer containing 0.2% glucose was used as suspension fluid.

Results. In Table I changes in the oxygen consumption of the cerebral cortex after thyroxine treatment are recorded. There seems to be some relation between duration of treatment and extent of increase in oxygen consumption.

Table II shows the influence of triiodothyronine on oxygen consumption, and also a comparison between the action of equal weight doses of thyroxine and triiodothyronine. The experiments show that triiodothyronine increases rate of brain respiration in newborn rats, but does not permit a conclusion that it has, in this case, a greater effect than thyroxine.

The influence of thyrotrophic hormone (TSH) on the brain cortex of newborn rats is shown in Table III. A dose of TSH which in 3 doses over 3 days seemed to produce little effect, appeared to produce a larger and statistically significant difference from the controls when given in 7 doses over 7 days.

The cerebellum of the newborn rat has normally a considerably higher oxygen consumption than that of the cortex. Thyroxine appears to raise the oxygen consumption (Table IV). The results however, are not significant owing to the great scatter in the single results.

Changes in the dry substance content of cortex, cerebellum and medulla region were investigated in 40 litters. A few representative results are given in Table V. This table shows that thyroxine, triiodothyronine and thyrotrophic hormone alike increase the dry substance content of all brain parts. Triiodothyronine does not appear to have a more marked action than thyroxine.

Discussion. It has been shown in these investigations that the oxygen consumption of the brain of the newborn rat can be increased under the influence of thyroid hormones. Thus it is considerably more sensitive to this treatment than the brain of the grown-up animal. It appears feasible that this action is equivalent to an acceleration of a physiological maturation process taking place in the brain. It is known(3) that the oxygen consumption of the brain rises during the first weeks of life.

It would be interesting to know why thyroid hormone acts as described on the brain of the newborn animal and not on that of the adult. It has been recently shown that the blood brain barrier is not yet established in newborn rats against glutamic acid(4) or Cl^{36}, or Thiocyanate(5). It would be tempting to assume that, similarly, during the first few days of life, thyroid substances can more easily contact the brain cells, owing to the fact that the blood brain barrier is not yet fully developed during this time.

Summary. 1. Thyroxine, triiodothyronine and thyrotrophic hormone significantly raise the brain oxidation of newborn rats; the dry substance content is also raised. These changes were regarded as an acceleration of normal maturation processes. 2. It is assumed that the described action of thyroid hormones on the brain of newborn animals is due to a still incompletely developed blood brain barrier.

1a. Hoexter, Frieda M., *Endocrinology*, 1954, v54, 1.

1. MacLeod, L. C., and Reiss, M., *Biochem. J.*, 1940, v34, 820.

2. Reiss, M., *Z. f. exp. Med.*, 1930, v77, 345.

3. Tyler, D. B., and van Harreveld, A., *Am. J. Physiol.*, 1942, v136, 600.

4. Himwich, H. E., and Himwich, W. A., in *Biochemistry of the developing nervous system*, Academic Press, 1955, p202.

5. Waelsch, H. in *Biochemistry of the developing nervous system*, Academic Press, 1955, p187.

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In vitro Effect of Soybean Phosphatides on Serum Lipoproteins of Normal and Hyperlipemic Subjects.* (22651)

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The oral administration of soybean lecithin has previously been reported to lower the serum cholesterol of man(1) and cholesterol-fed rabbits(2). The present study was initiated when preliminary experiments revealed that incubation of human serum, after addition of a fat emulsion prepared for intravenous administration, caused a shift of lipid from beta-globulin to alpha-globulin, as determined by paper electrophoresis. Because lecithin was used as a stabilizer in the fat emulsion, this phenomenon was investigated further using soybean phosphatides. The effects of not only the whole phosphatide complex, but also the alcohol-soluble and the alcohol-insoluble fraction were investigated.

Material and methods. 1. *Exp. A—Whole Phosphatide Complex.* A purified extract of natural soybean phosphatides† containing approximately equal quantities of lecithin, cephalin and lipositol was used as follows: Five mg of soybean phosphatides were dissolved in 1 or 2 ml of sera from 6 normal subjects in

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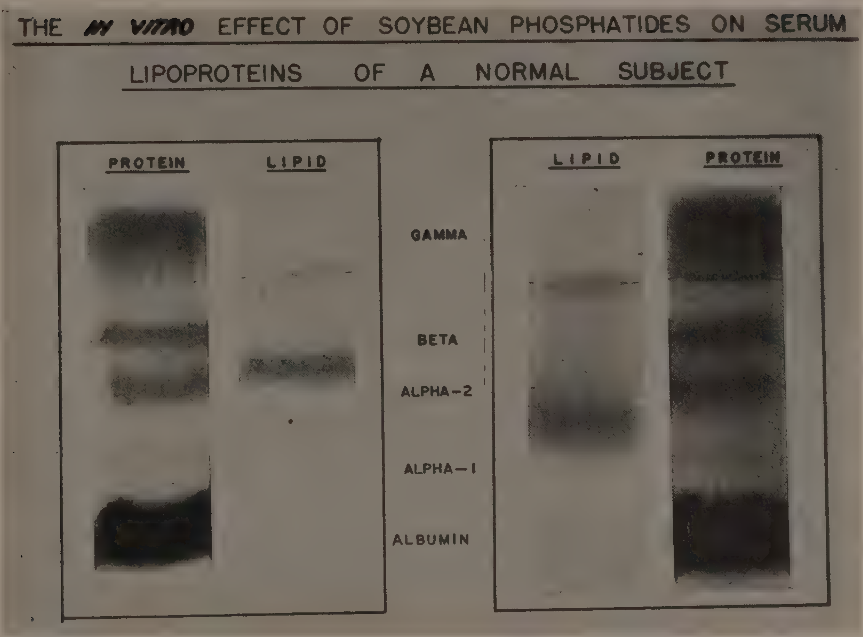
† This compound, Asolectin, was kindly supplied by Mr. J. Eichberg of the American Lecithin Co., Inc.


THE IN VITRO EFFECT OF SOYBEAN PHOSPHATIDES ON SERUM LIPOPROTEINS OF A NORMAL SUBJECT
PROTEIN
LIPID
GAMMA
BETA
ALPHA-2
ALPHA-1
ALBUMIN
LIPID
PROTEIN

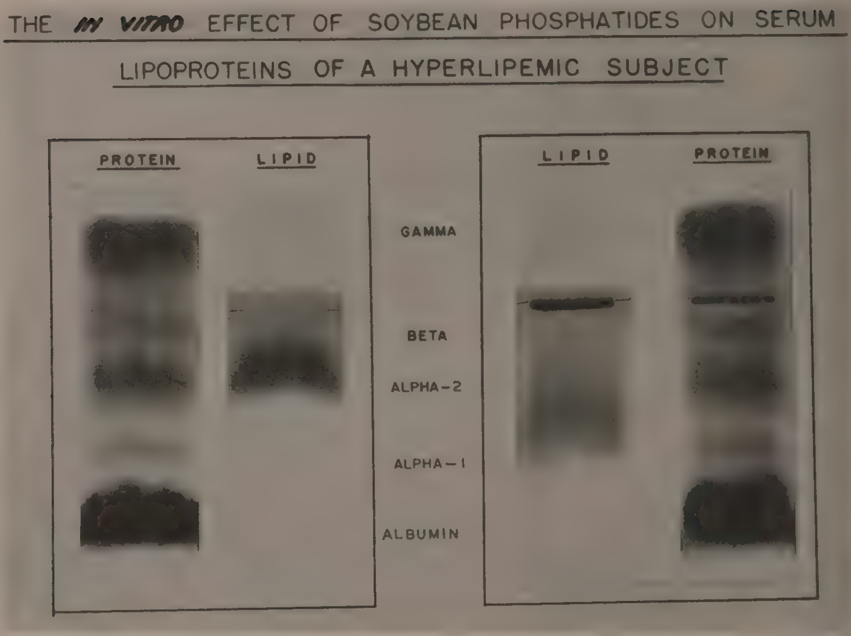
Control Phosphatide

FIG. 1. Effect of incubating the whole phosphatide complex with serum from a normal subject. Lipid stain is red; black and white reproduction does not permit good visualization of the faint alpha lipoprotein band.

the post-absorptive state. In a similar manner, 10 or 20 mg of the phosphatide complex were dissolved in the sera of 5 patients with hyperlipemia.‡ Serum aliquots without added phosphatide served as controls. The control sera and sera with added phosphatide were immediately streaked on filter paper strips for electrophoretic analysis, which was promptly performed with Flynn and deMayo's modification of Durrum's apparatus(3). The serum aliquots of 0.01 or 0.02 ml were streaked for protein or lipid staining respectively. The remaining control and experimental sera were incubated at 38°C for 24 hours, before being subjected to paper electrophoresis. Protein was stained with naphthalene black 12B200 and lipid with oil red O by technics previously described(4). Total lipids were determined gravimetrically (4), and lipid phosphorus by a modified Fiske-SubbaRow procedure(5) in aliquots of serum extracted with Bloor's reagent (alcohol 3 parts/anhydrous ether, 1 part). Cholesterol was determined by Bloor's method(6) on the total lipid residue.

2. *Exp. B-Alcoholic Fractions of the Phosphatide Complex.* The effects of the alcohol-soluble fraction of the soybean phosphatides, consisting of approximately ⅔ lecithin and ⅓ cephalin, and the alcohol-insoluble fraction, consisting of approximately ⅔ lipositol and ⅓ cephalin, on the serum proteins and lipoproteins of 10 normal and 9 hyperlipemic subjects were studied. In 1 ml of each normal serum, 5 mg of each fraction were separately dissolved. Similarly, in 1 ml of each hyperlipemic serum, 10 mg were dissolved. Samples of the resulting 4 mixtures and untreated control sera then were subjected to electrophoretic analysis both before and after incu-

‡ Early in these studies it was found that incubation with 5 mg of the phosphatide had no effect on migration in markedly hyperlipemic sera. When the phosphatide added was increased to 10 or 20 mg, changes in lipoprotein similar to that demonstrated in the normals were noted.


THE IN VITRO EFFECT OF SOYBEAN PHOSPHATIDES ON SERUM LIPOPROTEINS OF A HYPERLIPEMIC SUBJECT
PROTEIN
LIPID
GAMMA
BETA
ALPHA-2
ALPHA-1
ALBUMIN
LIPID
PROTEIN

Control Phosphatide

FIG. 2. Effect of incubating the whole phosphatide complex with serum from a hyperlipemic subject.

bation at 38 C for 24 hours. As in Exp. A, all sera were analyzed for total lipid, lipid phosphorus and total cholesterol prior to the experiment to determine degree of lipemia.

3. *Exp. C—Free Fatty Acids.* Free fatty acids were determined by the method of Grossman *et al.*(7) in aliquots of 7 sera incubated without phosphatide or with the alcohol-soluble or the alcohol-insoluble fractions. As in Exp. A, to 1 ml of each normal serum, 5 mg of either phosphatide were added whereas 10 mg were added to 1 ml of each of 2 hyperlipemic sera.

Results. 1. *Exp. A.* With the technic utilized, the lipid-staining band corresponding to beta lipoprotein normally is either associated with beta-globulin or is in an area between beta and alpha-2 globulin. This pattern was found in all the unincubated sera and in the phosphatide-free controls. However, in all of the phosphatide-containing incubated sera, both normal and hyperlipemic, the lipoprotein associated with beta-globulin migrated to the region between α-2 and α-1 globulin; *i.e.*, there was an increase in the migration velocity of beta-lipoprotein. (Fig. 1 and 2). In addition, in 5 of the 6 normal sera and 1 of the 5 hyperlipemic sera there was a definite but lesser increase in the migration of the lipid-staining band associated with α-1 globulin and albumin (alpha-lipoprotein) to an area on the strip immediately beyond albumin, after incubation with whole phosphatide complex.

2. *Exp. B.* The alcohol-soluble fraction produced little or no change in the serum lipoproteins after incubation. However, in all sera tested, incubation with the alcohol-insoluble fraction produced an increase in migration velocity similar to that demonstrated with the whole soybean phosphatide complex (Fig. 3).

3. *Exp. C.* Incubating sera with either the alcohol-soluble fraction or alcohol-insoluble fraction was associated with an increase in free fatty acid concentration, (Table I). However, no consistent differences between the 2 fractions on liberation of free fatty acids

were observed.

Discussion. In our previous unpublished preliminary investigation the same technic here described was utilized to test the effect of a fat emulsion containing 10% sesame oil and 4.5% dextrose stabilized with lecithin, on

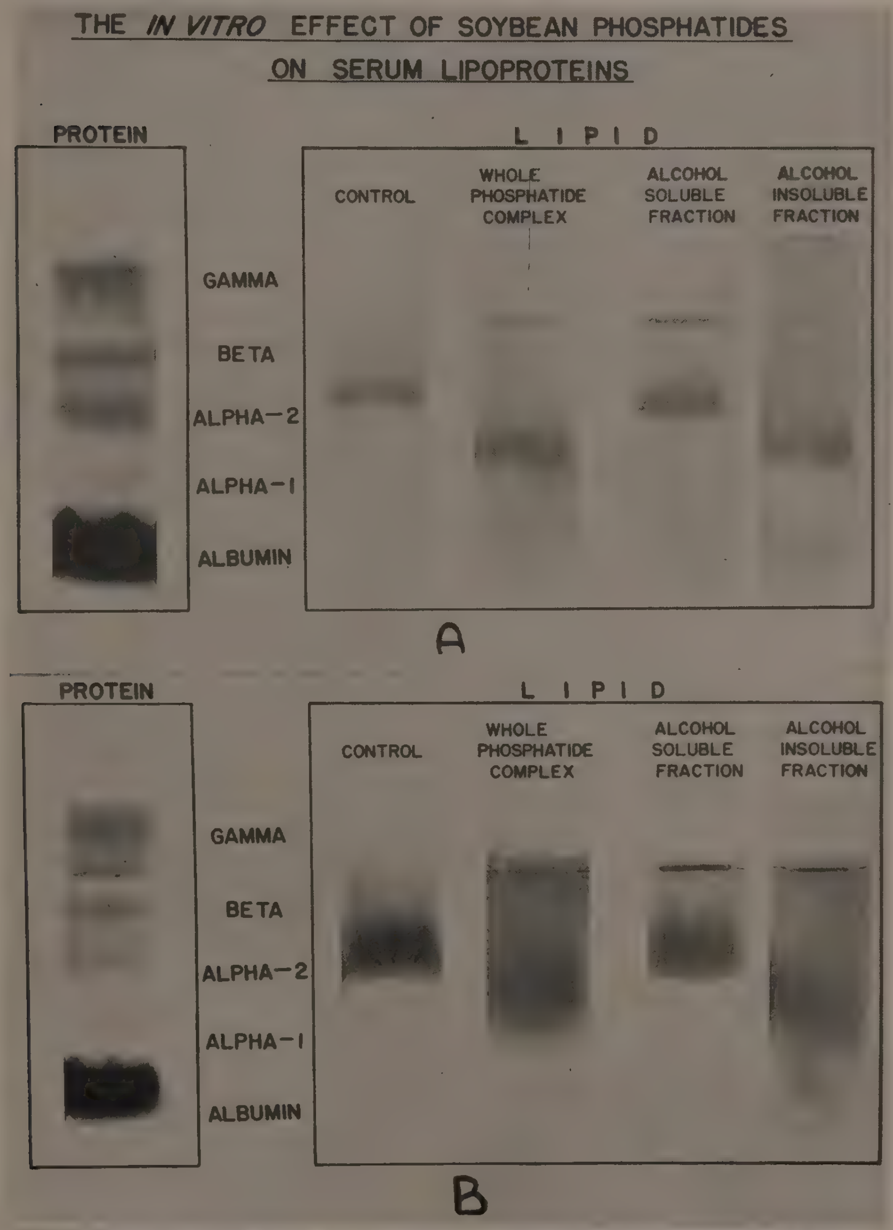

THE *IN VITRO* EFFECT OF SOYBEAN PHOSPHATIDES ON SERUM LIPOPROTEINS
PROTEIN
LIPID
CONTROL
WHOLE PHOSPHATIDE COMPLEX
ALCOHOL SOLUBLE FRACTION
ALCOHOL INSOLUBLE FRACTION
GAMMA
BETA
ALPHA−2
ALPHA−1
ALBUMIN
A
PROTEIN
LIPID
CONTROL
WHOLE PHOSPHATIDE COMPLEX
ALCOHOL SOLUBLE FRACTION
ALCOHOL INSOLUBLE FRACTION
GAMMA
BETA
ALPHA−2
ALPHA−1
ALBUMIN
B

FIG. 3. Comparison of effects of incubating the whole phosphatide complex and its 2 alcohol fractions on serum from (A) a normolipemic subject and (B) a hyperlipemic subject.

TABLE I. *In Vitro* Effect of Soybean Phosphatide Fractions on Serum Free Fatty Acids.

	Free fatty acids/ml of .02 N NaOH		
	Incubated serum		
Serum	+ Alcohol-soluble fraction	+ Alcohol insoluble fraction	Untreated control
A	.16	.12	.12
B	.20	.27	.12
C*	.22	.27	.11
D	.14	.13	.05
E	.13	.17	.07
F	.13	.13	.07
G*	.10	.12	0

* Hyperlipemic.

serum lipids and lipoproteins. The results were the same as the present observations with soybean phosphatide. As this paper was being prepared for publication, the lipid emulsion effect was confirmed *in vivo*(8). The observations on the effect of the lipid emulsion and the phosphatides are of particular interest because of the recent report by Lever and Waddell(9) that serum cholesterol falls following the intravenous administration of a fat emulsion, composed of 10% cottonseed oil and 1% of the same soybean phosphatide used in the present experiments. This preparation is the natural phosphatide complex, derived from soybean and consisting of 95% phosphatide with lecithin, cephalin, and lipositol present in approximately equal concentrations. It has been demonstrated that feeding soy lecithin produces significant falls in serum cholesterol levels of man(1,11,12), of cholesterol-fed rabbits(2) and, if soy sterols are also fed, of chicks(10). However, the effects of soybean phosphatides on serum lipoproteins have not been reported. The present comparison of the effects of the 2 fractions of the whole soybean phosphatide complex suggests that lipositol is probably the compound responsible for producing the lipoprotein shift. The alcohol-soluble fraction, (consisting of approximately ⅔ lecithin and ⅓ cephalin), produced little or no change in the serum lipoproteins after incubation. In contrast, the alcohol-insoluble fraction (consisting of ⅔ lipositol and ⅓ cephalin) produced a marked increase in migration velocity similar to that demonstrated with the whole soybean phosphatide complex. Simple addition of the whole phosphatide complex or either fraction, without incubation, produced little or no increase in the migration velocity. Lipositol is a lipid of unknown structural formula, which, on acid hydrolysis, yields inositol, phosphoric acid, oleic acid, saturated fatty acids, ethanolamine and tartaric acid. Alkaline hydrolysis of this inositol phosphatide liberates an amino-reducing carbohydrate, from which galactose may be obtained after mild hydrolysis(13). These effects produced by soybean phosphatide on the migration rates of serum lipoproteins are similar to those produced *in vivo* by heparin(14) or *in vitro* by "clearing factor"(15). *In vivo* heparinized plasma has been reported to cause a release of fatty acids from beta lipoproteins(16). Others(15,17) have found that fatty acids increase the electrophoretic mobility of alpha and beta lipoproteins *in vitro*. Therefore, soybean phosphatide might be increasing the migration velocities of lipoproteins by a similar mechanism; *i.e.*, by liberating fatty acids, too. However, the equivalent effects on free fatty acids following incubation with the alcohol-soluble as well as the alcohol-insoluble fractions suggest that some other mechanism must be responsible for the difference in the effects on lipoproteins produced by the two fractions.

Although several investigators(18-21) feel that an actual transformation of beta into alpha lipoprotein occurs, this position is disputed by others. Herbst *et al.* in recent studies,(22), which demonstrated that such an *in vivo* transformation does not occur, noted a pre-albumin component similar to that observed in some of the present sera and identified it as alpha lipoprotein. Sera used in their studies were obtained from subjects with either alimentary or idiopathic hyperlipemia. In the present series, the increase in migration velocities following soybean phosphatides was demonstrated on sera from fasting normolipemic as well as hyperlipemic patients.

Conclusions. Incubation of 6 normal and 5 hyperlipemic sera with a purified extract of natural soybean phosphatides produced an increase in migration velocity of beta lipoprotein, determined by paper electrophoresis. In 5 of the normal and one of the hyperlipemic sera increases in the migration velocity of alpha lipoprotein also were observed. Incubation of 10 normal and 9 hyperlipemic sera with the alcohol-soluble fraction of the phosphatide complex (which consists of ⅔ lecithin and ⅓ cephalin) produced little or no change in the migration velocities of the serum lipoproteins. Incubation of these sera with the alcohol-insoluble fraction, (which consists of ⅔ lipositol and ⅓ cephalin) produced an increase in the migration velocities similar to that noted with the whole phosphatide complex. Therefore, lipositol may be the compound responsible for the change observed. Determination of free fatty acids after incubation with either fraction failed to disclose any differential liberation of free fatty acids by the two fractions. The mechanism by which the alcohol-insoluble lipositol fraction increases the migration velocity of beta lipoprotein remains unexplained.

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1. Adlersberg, D., and Sobotka, H. J., *J. Mt. Sinai Hosp.*, 1943, v9, 995.

2. Kesten, H. D., and Silbowitz, R., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 71.

3. Flynn, E. V., and deMayo, P., *Lancet*, 1951, v2, 235.

4. Sachs, B. A., Cady, P., and Ross, G., *Am. J. Med.*, 1954, v17, 662.

5. Fiske, B. H., and SubbaRow, Y., *J. Biol. Chem*, 1925, v66, 375.

6. Bloor, W. R., *ibid.*, 1916, v24, 227.

7. Grossman, M. I., Palm, L., Becker, G. H., and Moeller, H. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 312.

8. Herbst. F. S. M., Lever, W. F., and Waddell, W. R., *Science*, 1956, v123, 843.

9. Lever, W. F. and Waddell, W. R., *J. Invest. Dermatol.*, 1955, v25, 233.

10. Peterson, D. W., Shneour, E. A., Peek, N. F., and Gaffey, H. N., *J. Nutrition*, 1953, v50, 191.

11. Steiner, A., and Domanski, B., PROC. SOC. EXP. BIOL. AND MED., 1944, v55, 236.

12. Pottenger, F. M., Jr., and Krohn, B., *Am. J. Digest. Dis.*, 1952, v19, 107.

13. Deuel, H. J., *The Lipids*, Interscience Publishers, 1951, v1, 450.

14. Rosenberg, I. N., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 751.

15. Laurell, S., *Scand. J. Clin. and Lab. Invest*, 1955, v7, 28.

16. Shore, B., Nichols, A. V., and Freeman, L. W., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.

17. Gordon, R. S., *J. Clin. Invest.*, 1955, v34, 477.

18. Boyle, E., Bragdon, J. H., and Brown, R. K., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 475.

19. Brown, R. K., Boyle, E., and Anfinsen, C. P., *J. Biol. Chem.*, 1953, v204, 423.

20. Williams, G. R., *Biochim. et Biophys. Acta*, 1954, v13, 72.

21. Nikkila, E., *Scandinav. J. Clin. and Lab. Invest.*, 1953, v5, Suppl. 8.

22. Herbst, F. S. M., Lever, W. F., Lyons, M. E., and Hurley, N. A., *J. Clin. Invest.*, 1955, v34, 581.

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Method for Study of Electrocardiogram of Early Chick Embryo within the Shell. (22652)

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The electric activity of isolated heart muscle of the chick embryo as an organ, or as a portion in tissue culture, has been studied by a number of investigators(1,2,4,5,6,7,10,11). Lagen and Sampson(8) employed this preparation, and Friedman and Bine(3) dissected the embryonic duck heart to study electrocardiographic changes in evaluating drugs. Today, isolated muscle from a number of animals is used in physiologic and pharmacologic studies for standardization of drug action upon the myocardium. All these technics involve considerable trauma and the use of artificial

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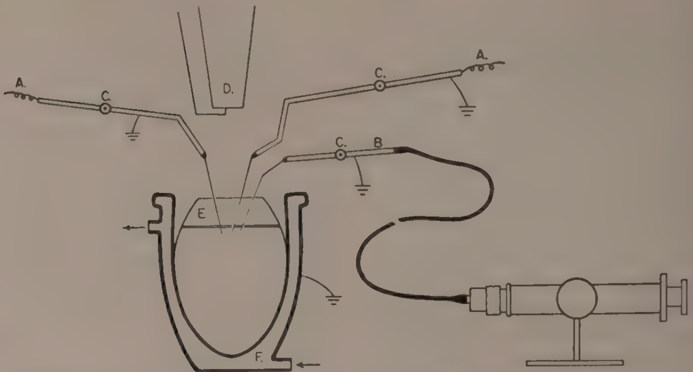

FIG. 1. Schematic drawing of the equipment. A—Connection from microelectrodes to recording apparatus; B—Insulated needle holder with ground connection; C—Universal ball type joint for needle holder; D—Dissecting microscope objectives; E—Egg air chamber; F—Egg holder with water jacket.

media with loss of spatial orientation of the fibers.

It is the purpose of this paper to describe a simple technic for obtaining electrocardiograms of early chick embryos without removal from the shell. Observations on the effect of drugs or of changes in temperature in such preparations can easily be made. The entire embryo remains in its natural environment, thus more physiological recordings can be expected.

Method. Medium sized Rhode Island eggs were incubated from 3 to 18 days at 103°F with daily rotations. From these, well developed embryos 3 to 16 days old were chosen. The eggs were mounted, air chamber on top, in a metal egg holder with a circulating water jacket (Fig. 1). The shell overlying the air sac was first sterilized with a 1% benzolkalium solution‡ and then removed, creating a window about 2 cm square. The mounted egg was placed under a dissecting microscope so that the tips of the recording electrodes were under direct vision. With incident light heart action was observed through the air sac membrane. Each electrode consisted of hypodermic stainless steel needles, one inch long, gauge #26, cut off at the hub and soldered to shielded copper wire. This was mounted in a special needle holder with ball joint (Fig. 1) to facilitate application to the selected area. The needles were insulated with varnish except for 3 mm at the tip.

To obtain maximum deflection in recordings from chick embryos 5 days old, electrodes were usually applied to the right wing and to the anterior wall of the chest. In older embryos, satisfactory recordings were obtained from the right wing, the left thigh or left wing. The needles were inserted deeply into the muscle and left in position during the period of observation.

When the embryos became quite active, as they are after 8 days of incubation, movement that interfered with the recordings was controlled by injection of 3:1000 mg curare or similar drugs.

Recordings were made on a Grass III D electroencephalograph by using EKG filter setting. The gain was adjusted to obtain a tracing of at least 7 mm deflection for the R wave. Following each recording, the instrument was calibrated with a standard value for the gain setting used. The recorder paper speed was 30 or 60 mm per second. When control tracings had been made, the drug to be tested was injected into an allantoic vein with a Pyrex glass microneedle, made in a De Fonbrune microforge and connected to an 0.01 cc tuberculin syringe. Electrocardiographic changes were recorded at various intervals. Throughout each test, a constant or

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‡ Winthrop Laboratories, 1450 Broadway, New York.

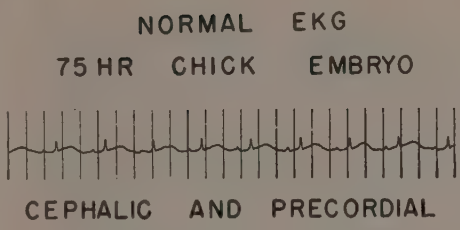

FIG. 2. R wave standard: 40 μvolts. Distance between vertical lines: 1/10 sec.

a variable temperature was maintained by means of the circulating water jacket to study temperature influence on heart action. All drug solutions were preheated before injection to the same temperature as that of the water jacket.

Results. Examples of the recordings obtained by this method from embryos at a constant temperature of 103°F are shown in Fig. 2, 3, and 4. Fig. 2 represents electrocardiograms of a chicken embryo 3 days old. At this age the P wave, the QRS complex and T wave are differentiated. The control tracing had a pulse rate of 240 beats per minute. Fig. 3 illustrates the control tracing in a 6-day-old chick embryo, and the same preparation 5 minutes after an injection of 0.1 cc of 1:10000 Ouabain (2.5 mg/cc) with heart block. Fig. 4 shows the control tracing from a 16-day-old chicken embryo. The control heart rate was 345 beats per minute. Sixty seconds after the injection of 0.2 cc of 1:1 billion Isuprel, the rate was 400 beats per minute.

Discussion. By maintaining the embryo in its natural environment, physiological conditions are maintained during recording of the EKG. The dissecting microscope permits correlation of direct heart observation with EKG changes. According to Lagen and Sampson(8) there is no nerve supply to the heart of the chicken embryo until the 6th day. Therefore, 3- and 4-day-old embryos provide a denervated heart preparation which may be valuable in the study of drug action when it is desirable to differentiate direct effect on the heart muscle. The use of chicken embryos within the shell to assay drugs presents definite advantages over methods currently employed, such as low cost, easy preparation, uniformity in quality, and the possibility of evaluating minute quantities of cardiac drugs. This testing procedure is also a suitable device for studying the effects of temperature changes upon the heart, and the action of drugs under hypothermia, since the time required for cooling and rewarming the entire chicken embryo is considerably less than that needed for larger animals.

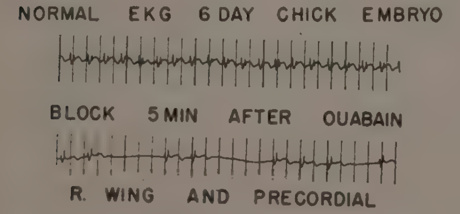

FIG. 3. R waves standard: 40 μvolts. Distance between vertical lines: 1/5 sec.

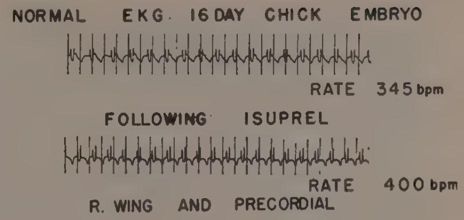

FIG. 4. R wave standard: 100 μvolts. Distance between vertical lines: 6/10 sec.

Summary. A method for the recording of electrocardiograms of the chick embryo within the shell after 3 to 16 days of incubation is described. Early results on evaluation of drug action upon the myocardium at different temperatures suggest, because of the simplicity and low cost, considerable advantages over present technics.

We wish to express our acknowledgement to Dr. J. S. Lieberman who contributed to the design of the electronic equipment.

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1. Armstrong, P. B., *J. Exp. Zool.*, 1931, v58, 43.

2. Ebert, J. D., Tolman, R. A., Mun, A. M., and Albright, J. F., *Ann. N. Y. Acad. Sc.*, 1955, v60, 965.

3. Friedman, M., and Bine, R., PROC. SOC. EXP. BIOL. AND MED., 1947, v64, 162.

4. Garb, S., and Chenoweth, M. B., *Circ. Res.*, 1953, v1, 135.

5. Garcia-Aust, S., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 348.

6. Diserens, H. W., *Am. J. Physiol.*, 1953, v172, 757.

7. Hoff, E. T., Kramer, D., Dubois, D., and Pattern, B., *Am. Heart J.*, 1939, v17, 470.

8. Lagen, J. B., and Sampson, J. J., PROC. SOC. EXP. BIOL. AND MED., 1932, v29, 735.

9. Olivo, O. M., *Atti Convegno Med., Giuliani*, Sept., 1946.

10. ———, *Atti Soc. Ital. Anat., Suppl.* 1947, 56.

11. Szepsenwol, J., *Anat. Rec.*, 1946, v95, 125.

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Some Observations on Nature of Refractoriness to Histamine Liberators.[*] (22653)

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The ability of a variety of agents including compound 48/80[†] to liberate histamine *in vivo* and *in vitro* has been well established. A phenomenon of acute tolerance development has been described for many of these agents and its mechanism is commonly ascribed to depletion of available body histamine stores by previous administration of the histamine liberator(1). The experiments here reported suggest the existence of an alternate mechanism of production of a refractory state to these histamine liberators.

Methods. Mongrel dogs were anesthetized with 33 mg/kg sodium pentobarbital. Arterial blood pressure was recorded from a common carotid artery and injections were made into a femoral vein. Serial blood samples were withdrawn in heparinized syringes from the other femoral vein in those experiments in which estimations of plasma histamine were made. Plasma histamine levels were determined by direct addition of the plasma to segments of guinea pig ileum suspended in atropinized Tyrode's solution, the contraction height being compared with those produced by known amounts of histamine. Pharmacologic identification of the stimulating agent as histamine was established by the inhibitory effect of Benadryl (final concentration 1:1x10^6).

Two methods of administration of compound 48/80 were employed: (A) 200 μg/kg compound 48/80 was administered as a single rapid injection. (B) A series of graded doses beginning with 1.5 μg/kg and increasing to 200 μg/kg compound 48/80 was administered by slow injections spaced 10 to 15 minutes apart (cumulative dose of 596 μg/kg over a 3- to 5-hour period).

Dogs thus treated were challenged with 200 μg/kg compound 48/80 injected rapidly, and in several instances by 0.1 ml/kg of 10% Tween 20.

Results. The precipitous and prolonged hypotensive effect of a rapid injection of 200 μg/kg compound 48/80 in the anesthetized dog (Fig. 1) qualitatively agrees with that

TABLE I. Plasma Histamine Concentrations before and after Administration of Compound 48/80.

		Plasma histamine conc. (μg/ml		
			Min. after 200 μg per kg compound 48/80	
Pre-treatment	No. of dogs	Control	5	15-30
None	3	.13 ± .04*	.57 ± .10	.40 ± .05
596 g/kg, compound 48/80 †‡	4	Not detectable§	Not detectable	—

* Mean ± stand. dev.

† This amount of compound 48/80 was administered over a period of 3-5 hr in a series of graded doses ranging from 1.5-200 μg/kg.

‡ No histamine was detectable in plasma samples obtained every 30 min. during this period.

§ Sensitivity of the histamine assay method is estimated to be .05 μg histamine.

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[*] This investigation was supported by research grant G-4077 (C) from the National Institutes of Health, Public Health Service.

[†] Compound 48/80 is a mixture of the dimers, trimers, and tetramers resulting from the condensation of N-methylhomoanisylamine and formaldehyde. Compound 48/80 was kindly made available to us by Dr. E. J. de Beer, The Wellcome Research Laboratories, Tuckahoe, N. Y.

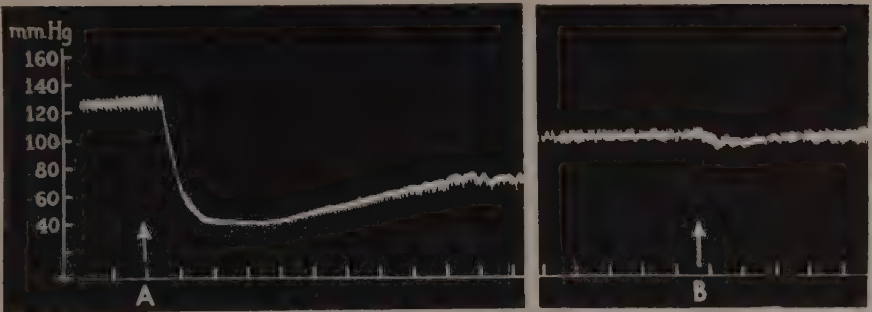

FIG. 1. Effect of compound 48/80 on blood pressure: At A and B, 200 μg/kg compound 48/80 was inj. rapidly. Base line signal interval, 1 min.; interval between sections, 69 min.

reported by others(2). An increase in plasma histamine concentration was found to occur in the early period of hypotension (Table I).

When the administration of compound 48/80 was modified to a series of graded doses ranging from 1.5 to 200 μg/kg, it was possible to inject a total of 596 μg/kg over 3 to 5 hours without producing changes in the blood pressure; these animals were subsequently refractory to the rapid injection of 200 μg/kg (Fig. 2). No changes in plasma histamine concentration were detectable during the period of administration of the graded doses or following the rapid injection of 200 μg/kg compound 48/80 in these animals (Table I).

Tween 20 produced a marked hypotensive effect in both groups of 48/80 refractory animals.

Discussion. The development of tolerance to the action of histamine liberators has been described in a variety of species. Feldberg and Paton(3) demonstrated the ability of various histamine liberators to deplete tissues of virtually all of their available histamine.

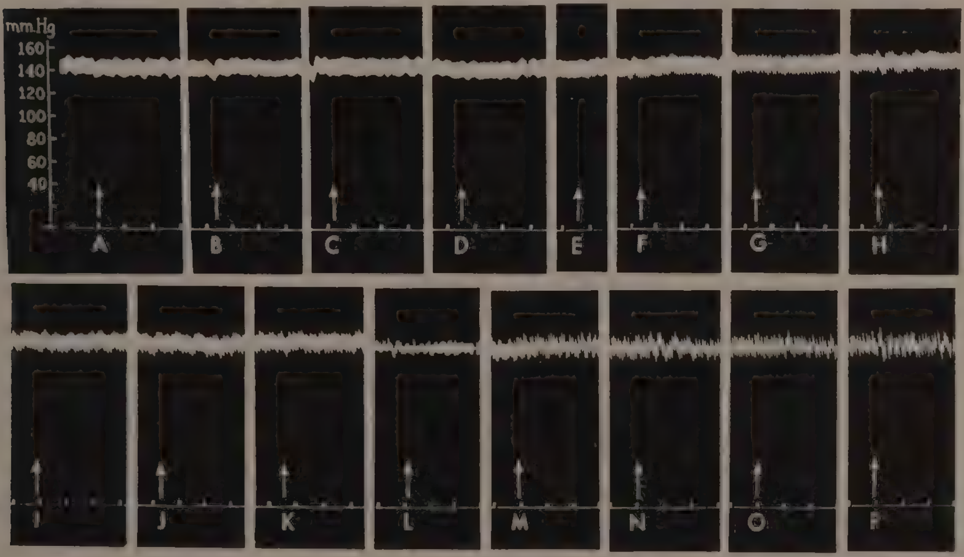

FIG. 2. Effect of compound 48/80 on blood pressure: Compound 48/80 was administered in a graded dosage schedule by slow injections as follows: At A and B, 1.5; C and D, 3.1; E and F, 6.3; G and H, 12.5; I and J, 25; K and L, 50; M and N, 100; O, 200 μg/kg. At P, 200 μg/kg was inj. rapidly. Base line signal interval, 1 min.; interval between successive injections, 5-10 min.

Sufficient histamine is released by compound 48/80 in the cat and dog to account for its hypotensive action, and refractoriness to this compound is associated with its inability to liberate more histamine(4). The experiments of Feldberg and Talesnik(1) indicate that refractoriness to compound 48/80 is associated with the depletion of available histamine body stores by prior administration of the histamine liberator. Mayeda(5) has demonstrated cross-tolerance to the histamine liberating action of peptone and sinomeminine, and Slomka and Goth(6) have demonstrated a similar cross-tolerance between compound 48/80 and several morphinan compounds. This cross-tolerance may also be explained on the basis of depletion of available histamine body stores. Indeed, the marked reduction in the edema produced by light in porphyrin-treated rats whose skin histamine had been markedly depleted by prior administration of compound 48/80(1) lends support to this hypothesis. Although depletion of available histamine stores provides an obvious mechanism for the development of tolerance to histamine liberators, the results of our experiments suggest that an alternate mechanism of inducing a refractory state exists. Since no increase in circulating histamine concentrations was detectable during the development of the refractory state to compound 48/80 with a graded dosage schedule, it appears unlikely that a significant amount of histamine was liberated, *i.e.* enough to explain the refractory state by depletion of the available histamine body stores. The responsiveness of the cardiovascular systems of such 48/80 refractory dogs to exogenous histamine is unaltered. The ability of the histamine liberator Tween 20 to produce its characteristic hypotensive effects in 48/80 refractory animals further suggests that the depletion of available histamine body stores has not been accomplished. The ability of compound 48/80(7) and Tween 20(8) to liberate histamine from liver mitochondria has been demonstrated.

Although its mechanism has not been elucidated, an interpretation of our results suggest that acute refractoriness to the histamine liberator compound 48/80 has been developed in dogs by means other than that of depletion of available body stores of histamine.

Summary. The administration of a series of graded doses of compound 48/80 produces a refractory state to the histamine liberating action of this compound. This refractory state appears to be effected by a means other than that of depletion of available histamine body stores.

1. Feldberg, W., and Talesnik, J., *J. Physiol.*, 1953, v120, 550-68.

2. Dews, P. B., *et al.*, *J. Pharmacol. and Exp. Therap.*, 1953, v107, 1-111.

3. Feldberg, W., and Paton, W. D. M., *J. Physiol.*, 1951, v114, 490-508.

4. Paton, W. D. M., *Brit. J. Pharmacol. and Chemotherap.*, 1951, v6, 499-508.

5. Mayeda, H., *Jap. J. Pharmacol.*, 1954, v3, 73-81.

6. Slomka, M. B., and Goth, Andres, *J. Pharmacol. and Exp. Therap.*, 1956, v116, 52.

7. Grossberg, A. L., and Garcia-Arocha, H., *Science*, 1954, v120, 763-4.

8. Goth, Andres, unpublished results.

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Tissue Distribution of Parenteral Co^{60} Vitamin B_{12} in Mouse, Hamster, Rat and Guinea Pig.* (22654)

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(Introduced by B. S. Walker.)
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Species differences in the tissue distribution of parenterally administered Co^{60} vit B_{12} ($Co^{60}B_{12}$) have been suggested by recent studies. In the rat, the kidneys were found to be the main site of $Co^{60}B_{12}$ localization even at a time when urinary excretion was absent(1,2,3). In man, the greatest percentage of a parenterally injected dose of $Co^{60}B_{12}$ was found in the liver(4). In the chick(5) minimal localization of $Co^{60}B_{12}$ was found in the liver and kidney. In this study parenteral $Co^{60}B_{12}$ was administered to hamsters, rats, mice and guinea pigs in order to elaborate on possible species differences in its tissue distribution.

Methods. Experimental plan: Eight adult male Swiss white mice weighing 25-40 g, 8 adult hamsters (4 male and 4 female) weighing 85-120 g, 7 adult male Sprague-Dawley rats weighing 200-230 g, and 4 adult male guinea pigs weighing 450-600 g, were subcutaneously injected under ether anesthesia with $Co^{60}B_{12}$ (secured from Merck and Co., Rahway, N.J.). The dose was contained in a volume of 1.0 ml, except for mice, where 0.5 ml was given. The dose injected ranged from 0.10 to 0.40 μc of Co^{60}. Mice received 0.15 μg of B_{12}, hamsters 0.70 μg, rats 0.30 μg and guinea pigs 0.26 μg. After injection, rats and guinea pigs were housed in metabolic cages so as to allow separate urine and stool collections. Hamsters were housed in wire cages under which 2 thicknesses of Whatman filter paper #3 were spread. All stools fell on the filter paper while the urine was absorbed into the paper. Mice were housed in 2,000 ml beakers. The bottoms of the beakers were also lined with this filter paper on which urine and stools were deposited. Four days after the injection of the Co^{60} B_{12}, animals were anesthetized, exsanguinated by cardiac puncture, and the appropriate organs removed.

Preparation of specimens for analysis: (a) *Organs.* The liver, kidneys, spleen, heart, testes and portions of the hind leg musculature were removed and weighed. Organs were placed into flasks containing 10 ml of 2N NaOH, except for the livers where 20 ml was used. The organs were digested in alkali at room temperature for 24 hours. Then, if necessary, gentle heating for 30 to 60 seconds brought the gel-like digest into a liquid state. Measurements of radioactivity were made on duplicate 4 ml aliquots of these tissue digests. The total volume of the tissue digest solution was assumed to be equal to 10 ml when organ weight was less than 0.75 g as the volume contributed by the dissolved organ was negligible. For heavier organs the total volume of the digest was measured in a 25 ml graduated cylinder. (b) *Urine, stools, and blood.* Independent total urine collections were secured for the first 24 hours and for the following 3 days for the rats and guinea pigs. The total urine volume was measured in a 50 ml graduated cylinder. Radioactivity was determined on 4 ml aliquots of urine. For hamsters and mice, the filter paper on which the urine had dried was cut into small pieces and placed into 1¾ by 3¼ inch glass bottles in which radioactivity was measured. The radioactivity in 4-day stool collections was also determined in 1¾ by 3¼ inch glass bottles. One to 4 ml of oxalated blood (hamsters and rats were only done) was used for radioactivity assay. (c) *Carcasses.* Mice and hamster carcasses were placed into 1¾ by 3¼ inch glass bottles while the rat carcasses were placed into 2½ by 6 inch glass bottles. Car-

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* This work was supported in part by grants from the United States Public Health Service and the Atomic Energy Commission.

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‡ Present address: University of California Medical Center, Los Angeles.

TABLE I. Tissue Distribution and Excretion of $Co^{60}B_{12}$ in Mice, Rats, Hamsters and Guinea Pigs.

	Mice		Rats		Hamsters		Guinea pigs	
Organ	% of inj. dose*	% of dose* / g wet wt	% of inj. dose	% of dose / g wet wt	% of inj. dose	% of dose / g wet wt	% of inj. dose	% of dose / g wet wt
Kidney	6.2 ± .4	13.0 ± 1.4	16.2 ± 2.9	9.7 ± 1.9	7.2 ± .6	6.3 ± .1	2.4 ± .1	.3 ± 0
Liver	23.7 ± 2.5	11.2 ± 1.1	7.4 ± .4	.8 ± 0	18.0 ± 2.9	6.1 ± .1	29.7 ± 4.1	1.3 ± 0
Testes	1.5 ± .1	7.0 ± 1.1	1.0 ± 0	.6 ± 0	not done	—	.3 ± 0	.1 ± 0
Spleen	1.4 ± .2	1.7 ± .5	.8 ± 0	.6 ± 0	.3 ± 0	.8 ± 0	.2 ± 0	.3 ± 0
Heart	.4 ± .1	1.8 ± .1	.7 ± 0	.9 ± 0	.4 ± 0	.6 ± 0	.2 ± 0	.1 ± 0
Muscle	15.9 ± .9	1.3 ± 0	13.6 ± 1.1	.2 ± 0	7.7 ± 1.5	.3 ± 0	not done	—
Carcass	26.0 ± 2.9	—	34.5 ± 4.0	—	43.0 ± 3.8	—	" "	—
Urine	5.9 ± 1.8	—	7.4 ± 1.1	—	14.1 ± 1.0	—	12.5 ± .9	—
Stool	15.5 ± 1.8	—	18.5 ± 6.2	—	3.1 ± .9	—	5.5 ± .1	—
Recovery	106.1		100.7		95.1		50.8	

* Mean and stand. error of mean.

cass radioactivity measurements were made in these bottles. Because of their large size, the guinea pig carcasses could not be counted.

Radioactivity determination: A 1:100 dilution of the injected $Co^{60}B_{12}$ was made and varying aliquots were then used as standards for the varying conditions employed in the counting of organ digests, excreta and carcasses. By adding water as needed, the height of the standard in the glass bottles was always the same as that of the specimen being counted *i.e.*, stools, urine or carcasses. Blood, tissue and liquid urine samples were counted in a scintillation well-type counter (thallium activated sodium iodide scintillation crystal). The radioactivity of the organ samples usually counted 3-12 times background, except for the muscle, heart, blood and spleen which usually counted 1-3 times background. Samples were counted long enough to give a counting error of less than 2%, except for the slow-counting samples where a 5-8% counting accuracy was obtained. Duplicate samples usually agreed within 1-5%. All samples contained in the 1¼ by 3¼ inch glass bottles were counted by placing them on top of the above-mentioned sodium iodide well crystal. The diameter of these was just about equal to that of the diameter of the well. Under similar geometry, a standard was also counted. The 6 inch bottles containing the rat carcasses were marked into 3 equal 2 inch segments. With the bottle in the horizontal position, and directly in contact with a solid 1 by 1 inch thallium activated sodium iodide crystal, each 2 inch segment of the bottle containing the carcass was counted. Under similar geometry the bottle containing the standard was counted.

Calculations. (a) The percent radioactivity in organ =

$$\frac{\text{cpm per ml organ digest x total volume digest}}{\text{cpm injected}} \text{ x } 100$$

In calculating the total percent uptake in the muscle mass, 40% of the animal's weight was assumed to be muscle. For the total percent uptake in blood, a blood volume was estimated based on the figure of 69 ml kg body weight.

Results. The organ distribution and excretion of a parenteral dose of $Co^{60}B_{12}$ in the rat, guinea pig, mouse and hamster are shown in Table I.

Discussion. In the rat the tissue distribution of a parenterally injected dose of $Co^{60}B_{12}$ was similar to that found by Rosenblum *et al.* (1). Similar studies on the mouse, hamster and guinea pig are not available in the literature. Differences in the major organ sites of $Co^{60}B_{12}$ localization were noted in the 4 species studied. In the hamster, guinea pig and mouse, the liver was the chief organ of localization whereas in the rat the kidney was the chief site. The high $Co^{60}B_{12}$ uptake of the rat kidney is probably unrelated to its known role in the excretion of large doses of parenterally injected vit B_{12}(3,6,7), since the dose given the rat was comparable to that given the other 3 species. Furthermore, the other 3 species studied had comparable amounts of radioactivity excreted in the urine without an increase in kidney $Co^{60}B_{12}$ uptake. At the time the high rat renal $Co^{60}B_{12}$ uptake was found, urinary excretion of the radiovitamin was negligible making it highly unlikely that the $Co^{60}B_{12}$ in the kidneys was there to be excreted. This is further supported by the observations of Harte(2), who found that high renal $Co^{60}B_{12}$ levels were maintained for as long as 90 days after injection despite the absence of urinary excretion after the first day. It would thus appear that the kidney represents an important site of $Co^{60}B_{12}$ localization in the rat rather than a mere storage site for further urinary excretion. The high $Co^{60}B_{12}$ concentration in the liver of the mouse, hamster and guinea pig is comparable to that found in man after the injection of labelled vit B_{12}(4). The rat, with the chief localization of injected $Co^{60}B_{12}$ in the kidney, is not analogous to man. It would, therefore, appear hazardous to relate to man the results of the experiments in the rat.

Differences in the routes of $Co^{60}B_{12}$ excretion were noted in the 4 species studied despite the administration of comparable amounts of vit B_{12} in relation to body weight. The stools were the chief routes of excretion in the rat and mouse, whereas the urine was the chief excretory pathway in the hamster and guinea pig. In previous studies in the rat (1,8) the urine was the major excretory pathway containing 50-90% of a parenteral dose of $Co^{60}B_{12}$. These results differ markedly from those found in this study and are probably due to the large doses of vit B_{12} (4-20 μg) used in these studies. There is no available data on the excretion of parenteral $Co^{60}B_{12}$ in the other 3 species studied. The present investigation suggests that the stools may be an important route for the excretion of vit B_{12} in the rat and mouse. Radioactivity as measured in this study merely indicates the presence of the Co^{60} moiety of the vit B_{12} molecule. Thus, the radioactivity may be due to vit B_{12} or to an intermediate degradation product of the vitamin. After parenteral administration of $Co^{60}B_{12}$ to rats, the radioactivity excreted in the urine was found to be due to vit B_{12}, whereas that in the stool was unassociated with vit B_{12} activity(1). The radioactivity in the kidneys was found to be due to vit B_{12} in a similar study(8). It appears likely that the radioactivity in the kidneys in this study is due to vit B_{12}. However, concerning the other organs, this question cannot be answered.

Summary. The excretion and tissue distribution of parenterally administered Co^{60} B_{12} has been studied in the rat, hamster, mouse and guinea pig. The liver contained more radioactivity than any other organ in the hamster, mouse and guinea pig, whereas the kidneys contained more radioactivity than any other organ in the rat. In the rat and mouse, more radioactivity was excreted in the stools than in the urine whereas in the guinea pig and hamster, the urine was the chief excretory pathway.

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1. Rosenblum, C., Chow, B. F., Condon, G. P., and Yamamoto, R., *J. Biol. Chem.*, 1952, v198, 915.

2. Harte, R. A., Chow, B. F., and Barrows, L., *J Nutr.*, 1953, v49, 669.

3. Yamamoto, R., Barrows, C., Lang, C. A., and Chow, B. F., *ibid.*, 1951, v45, 507.

4. Glass, G. B. J., Boyd, L. J., and Gellin, G. A., *Blood*, 1955, v10, 95.

5. Monroe, R. A., Patrick, H., Comar, C. L., and Goff, O. E., *Poultry Sci.*, 1952, v31, 79.

6. Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C., and Lang, C. A., *J. Lab. Clin. Med*, 1951, v38, 84.

7. Sokoloff, M. F., Sanneman, E. H., and Beard, M. F., *Blood*, 1952, v7, 243.

8. Barbee, K. W., and Johnson, B. C., PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 720.

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Multiple Intracellular Recording from Atrial and Sino-atrial Cells: Correlation with Contractile Tension.* (22655)

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(Introduced by T. A. Loomis.)
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In the course of microelectrode studies of rabbit atria in this laboratory it was noted that cells in the region of the sino-atrial node failed to respond to as high frequencies of electrical stimulation as did atrial cells. The present study was undertaken to investigate this phenomenon by means of simultaneous recording of atrial and sino-atrial cell action potentials. An effort was made to correlate the contractile response with the electrical activity of atrial muscle.

Methods. Virgin female albino rabbits were sacrificed by a blow on the neck and quickly exsanguinated. The thorax was opened and the entire heart quickly removed and dropped into oxygenated Tyrode solution, as modified by Hoffman and Suckling(1). In this solution, through which oxygen (95%)-carbon dioxide (5%) constantly bubbled, the combined atria were carefully separated from the ventricles and then mounted in a Plexiglas bath containing Tyrode solution. The bath was oxygenated through a diffusion dish which was incorporated into the chamber. The nutrient solution constantly flowed through at a rate of 3 cc per minute. The right atrial appendage was fastened to 2 silver hooks which also served as the stimulating electrode. The connective tissue in the septal area was secured to the arm of a sensitive strain gauge (Grass force displacement transducer), and the left atrium was allowed to float freely in the chamber. Stimulation was applied by means of a Grass Model 3C Stimulator. The output was passed through an isolation transformer to reduce the shock artifact. The signal produced by distortion of the strain gauge arm during contraction, was fed into a preamplifier, which in turn supplied the input to a DC amplifier of a Sanborn 4 channel recorder. Transmembrane potentials were determined with two cathode follower circuit units(2), using simultaneously 2 glass capillary electrodes with tip diameters of 0.5 μ or less. One electrode was used in the atrial myocardium exclusive of pacemaker region, while the other was used to explore the pacemaker area. Each of these circuits was connected to a separate DC amplifier in a Sanborn 4 channel recorder. The temperature of the bath was maintained between 28-30°C in order to reduce the spontaneous rate, lengthen the duration of the action potential and to maintain better contractility.

Results. Tracings of typical records obtained during simultaneous impalement of atrial and sino-atrial cells are seen in Fig. 1. The dotted line represents the zero potential level. Sino-atrial cell firing can be recognized by the slow diastolic prepotential development as noted in the upper portion of the tracings. After a control period, electrical stimulation was initiated and maintained for 1 to 3 seconds. Recovery represents the beginning of spontaneous activity following electrical stimulation. At the 2 frequencies depicted (3.75 and 4.6 stimuli per second), the atrial cell follows, while the sino-atrial cell follows at the lower but not at the higher frequency. In general, the amplitude of atrial cell action potentials was 90-120 mv, while that of sino-atrial cells was lower (50 to 70 mv). The lower values for sino-atrial cell action potentials are in agreement with the reports of other investigators working with rabbit atria(2) and frog sinus venosus(3,4).

Fig. 2 summarizes the results obtained on frequency of response of atrial and sino-atrial cells versus frequency of electrical stimulation applied. Blackened circles (sino-atrial responses) and open circles (atrial cell responses) represent the arithmetic mean of 2

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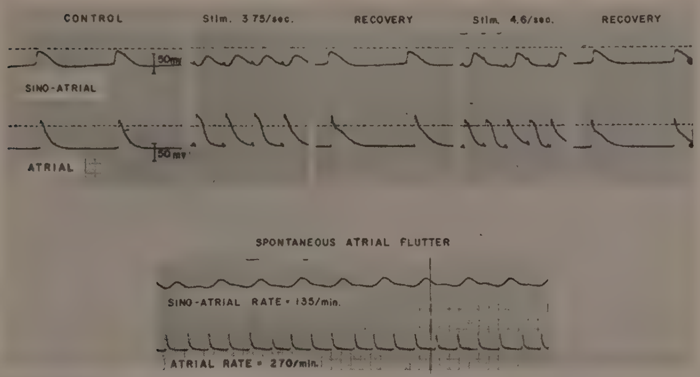

FIG. 1. Simultaneous recording of sino-atrial and atrial cell action potentials during electrical stimulation and spontaneous atrial flutter. Dotted line represents zero potential level. Each small division represents one mm. Paper speed, 50 mm/sec.

to 18 determinations. At lower frequencies the minimum number of determinations was 4 to 5, with a maximum at 18, while at higher frequencies (8-16 stimuli per second) a minimum of 2 determinations were made. The vertical bars are ranges of responses at any one frequency. Inspection of the graph discloses a linear relationship (1:1) for atrial cells at stimulation frequencies as high as 10 per second. The slope of the line drawn through the open circles is unity. Sino-atrial cells fail to follow rates as low as 4 per second. Stimulation frequencies beyond this point resulted in an integral ratio of atrial to sino-atrial cell responses; being 2:1 up to a frequency of 7.5 per second, then 3:1 and 4:1 at higher frequencies.

A recording taken at a time of spontaneous atrial flutter is shown in Fig. 1. There is a 2:1 ratio between atrial and sino-atrial cell firing, a phenomenon which parallels the results obtained from electrical stimulation.

Shown in Fig. 3 are simultaneous recordings of action potentials of an atrial cell and the tension developed by the right atrium. At the onset of electrical stimulation there is a decrease in tension (3rd beat, Fig. 3). This has been reported for isolated papillary muscle by Gold and Cattell(5). As stimulation was continued, the tension alternated in a regular manner such that a beat in which high tension developed was followed by a beat of low tension. Both the high and low tension contractions exhibited a positive staircase effect(6,7) during the applied electrical stimulus. When stimulation was stopped, there was a marked increase in tension on recovery with the occurrence of a negative staircase effect. It is of interest to note the repolarization phase of atrial cell action potentials during electrical stimulation (4th to 6th and 9th to the 13th beats in Fig. 1, 3rd to the 12th beats in Fig. 3). Alternation in repolarization configuration can be seen; every other beat exhibited an exponential repolarization in contrast to the more sigmoid repolarization of the intervening beats. In Fig. 3, alternation becomes apparent during continued electrical stimulation. The tension is greater in those cycles where repolarization

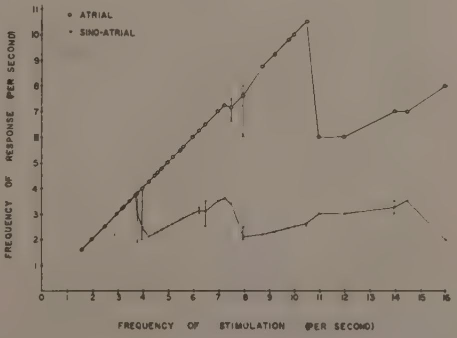

FIG. 2. Graph summarizing data of simultaneous recording of atrial and sino-atrial cell action potentials during electrical stimulation. Open circles, atrial cell responses; blackened circles, sino-atrial cell responses. Ordinate: frequency of electrical stimulation. Abscissa: frequency of response.

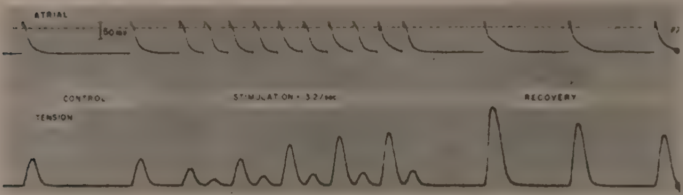

FIG. 3. Simultaneous recording of atrial cell action potentials (upper tracing) and tension developed by right atrium (lower tracing). Dotted line represents zero potential level. Each small division represents one mm. Paper speed, 50 mm/sec.

was exponential rather than sigmoid (5th, 7th, 9th and 11th beats in Fig. 3). Post-stimulation potentiation of tension is accompanied by a repolarization curve which is considerably longer in duration than those of the control action potentials or those occurring during electrical stimulation (7th and 15th beats in Fig. 1, 13th beat in Fig. 3). Tension development and action potential configuration returned to control values after 4 or 5 spontaneous beats.

Discussion. The difference in the follow rates of sino-atrial and atrial cells is to be expected on the basis of the much longer duration of the action potentials of sino-atrial cells(2,8,9). An electrical stimulus falling during the earlier phases of repolarization fails to excite sino-atrial cells, while on the same time scale the stimulus would excite atrial cells which have already returned to the polarized state. The sino-atrial region appears to act as pacemaker normally because it is usually in a partially depolarized state (2,8,9) and has the greatest tendency to undergo cyclic spontaneous depolarization. Certainly, pacemaker activity cannot be explained on the basis of refractory period, since the experiments reported here indicate a longer refractory period for sino-atrial cells. The same discrepancy between atrial and sino-atrial cells has been observed when a relatively rapid "flutter" has been induced in the preparation. When the rate of stimulation exceeded that rate at which the sino-atrial cells could follow, it was observed that alternation in both electrical and mechanical phenomena occurred. That is, the repolarization of atrial cells alternated between a sigmoid and exponential curve. At the same time, total contractile tension alternated in magnitude. It was observed that high tension was associated with the exponential type repolarization curves whereas low tension matched the sigmoid type repolarization. Inspection of simultaneous tracings of atrial and sino-atrial spikes showed that effective depolarization in sino-atrial cells also was coincidental with the exponential repolarization curve in atrial cells. In those experiments in which simultaneous atrial and sino-atrial electrical events and contractile tension all were observed, the same correlation was observed within limits. The cause-effect relationship is not yet clear. However, at higher frequencies a 4:1 ratio between atrial and sino-atrial response may be evident without a similar pattern in tension magnitude. It is felt that the correlation between sino-atrial cell firing and tension, when tension is alternating at lower frequencies, probably is coincidental. On the other hand, the relationship between atrial cell repolarization and tension, which is a consistent finding in this preparation, appears to be valid at all frequencies, although they are not related in any simple way. Further investigation of this phenomenon is in progress.

Post-stimulation potentiation and post-extrasystolic potentiation in the heart have been studied by other investigators. The studies of Rosin and Farah(10) were conducted on isolated rabbit atria and ventricles; those of Furchgott and Sleator(11) on guinea pig atria. Changes in contractility were qualitatively similar to those reported here. Neither of these authors attempted to relate contractility to transmembrane potentials. This has been done recently by Hoffman, Bindler and Suckling(12) using cat papillary muscle. However, the post-extrasystolic potentiation observed by the latter investigators was not accompanied by a significant change in membrane repolarization.

Summary. 1. Cells in the region of the sino-atrial node of rabbit are unable to respond to as high a frequency of stimulation as those cells not included in the pacemaker area. 2. The same phenomenon has been observed during rapid atrial arrhythmias in the absence of electrical stimulation. 3. The magnitude of the contractile tension developed by the atrium is related to repolarization of atrial cell action potentials, being greater for those demonstrating exponential rather than sigmoid curves. This is a consistent finding occurring during periods of electrical stimulation and with the first few spontaneous beats following stimulation, but the tension and action potential repolarization are not related to each other in any simple way.

1. Hoffman, B. F., and Suckling, E. E., *Am. J. Physiol.*, 1953, v173, 312.

2. West, T. C., *J. Pharmacol. and Exp. Therap*, 1955, v115, 283.

3. del Castillo, J. and Katz, B., *Nature*, 1955, v175, 1035.

4. Hutter, O. F., and Trautwein, W., *ibid.*, 1955, v176, 512.

5. Cattell, McK. H., and Gold, H., *Am. J. Physiol.*, 1941, v133, 236.

6. Szent-Györgyi, A., *Chemical Physiology of Contraction in Body and Heart Muscle*, New York, Acad. Press, 1953.

7. Hajdu, S., *Am. J. Physiol.*, 1953, v174, 371.

8. Arvanitaki, A., *Properties rhythmiques de la matiere vivant. II. Etude experimentale sur le myocarde d'helix.* Hermann, Paris, 1938.

9. Brady, A. J., and Hecht, H. H., *Am. J. Med.*, 1954, v17, 110.

10. Rosin, H., and Farah, A., *Am. J. Physiol.*, 1955, v180, 75,

11. Furchgott, R. F., and Sleator, W., *J. Pharmacol. and Exp. Therap.*, 1954, v110, 20.

12. Hoffman, B. F., Bindler, E., and Suckling, E. E., *Am. J. Physiol.*, 1956, v185, 95.

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Effect of DPPD, Methylene Blue, BHT, and Hydroquinone on Reproductive Process in the Rat.* (22656)

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Although several compounds have been found to prevent or cure certain vit. E deficiency symptoms, such as brown discoloration of the uterus and cloudy swelling of the convoluted tubules of the kidney, only 2, diphenyl-p-phenylenediamine (DPPD) and methylene blue, have been reported to substitute for tocopherol in preventing resorption-gestation in vit. E-deficient female rats. In our laboratory also, preliminary experiments indicated that methylene blue and DPPD gave slight positive responses under the conditions of our standard vit. E bioassay procedure(1). Consequently, we were led to investigate this apparent vit. E activity further.

Methylene blue. The results of vit. E bioassays in which the responses of 5 levels of methylene blue were compared with five levels of standard, *d*-α-tocopherol. showed median fertility doses (MFD) of 36 mg and 0.29 mg respectively. Thus, methylene blue has less than 1% the activity of α-tocopherol. However, during the course of these experiments, Moore, Sharman, and Ward(2) reported that under the conditions of vit. E bioassay in their laboratory, methylene blue was completely negative. The bioassay procedures used at Cambridge and by us were practically identical except for length of time of depletion. Moore used 5 months and we used about 5 weeks. Consequently, the slight positive response we obtained for methylene blue may have been due to its protective or sparing effect on the small, residual quantities of α-tocopherol still remaining in the tissues of our animals after only 5 weeks deple-

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* Communication No. 233.

TABLE I. Effect of DPPD* and *d*-α-Tocopheryl Acetate on Reproductive Performance of Vit. E-Deficient Female Rats.

	Dietary supplement		
	Negative control, no suppl.	0.2% DPPD	0.002% *d*-α-tocopheryl acetate
Fertility index (No. of females pregnant to No. mated)	3/3	9/9	9/9
Litter efficiency (% of pregnant animals with at least 1 viable fetus)	0	33	100
Mortality (No. of females dying at parturition)		2	0
Mean length of gestation (days)		25	22
Mean litter size		2.3	7.8
Viability index (No. of young alive at 3 days to No. born)		0/7	63/70
Lactation index (No. of young weaned to No. alive at 3 days)		—	61/63

* Feed grade DPPD.

tion. Quite recently, Christensen, Dam, and Gortner(3) revised their original conclusion (4) since methylene blue has been found to be ineffective as vit. E in preventing fetal resorption if the depletion diet is rigorously freed of tocopherol, thus eliminating the sparing effect of methylene blue on small, residual amounts of tocopherols in the vit. E-low ration. Therefore, methylene blue is not a vit. E substitute for preventing fetal resorption and, furthermore, should be used with caution, according to Moore(5), since at high levels of 0.252% in the diet it caused growth depression and severe damage (fragmentation) to the spleen of male rats. We also observed that all female rats died when supplemented with 100 mg/day of methylene blue.

DPPD. Results of a standard bioassay of DPPD in a Tween-water dispersion for vit. E potency showed that under the conditions of our test (5-12 week depletion period), DPPD possessed 7% the activity of the *d*-α-tocopherol standard on a molar basis. Johnson and Goodyear(6) reported that DPPD (0.385 mg/wk) supported reproduction in female rats maintained on a vit. E-low ration from weaning. Subsequent experiments showed(7) that this vit. E-like effect of DPPD disappeared as soon as the animals' tissue stores of tocopherol were used up.

A further test of the apparent anti-sterility potency of DPPD was conducted in which DPPD was fed mixed into the vit. E-low diet and fed to vit. E-deficient female rats beginning 7 days prior to mating. In Table I are shown the results obtained in comparison with a control group treated identically except that the diet was supplemented with 0.002% *d*-α-tocopheryl acetate instead of DPPD. DPPD in the diet (0.2%) was less effective than *d*-α-tocopheryl acetate (0.002%) in preventing typical vit. E-deficiency gestation resorptions, as evidenced by "litter efficiencies" of 33% and 100%, respectively. But more striking was the effect of DPPD on the duration of gestation and on mortality of young. Gestation was prolonged to 25 days from a normal of 22 days, and all of the newborn rats died before 3 days of age.

Other experiments with a vit. E-free diet or a stock diet confirmed these adverse effects of DPPD. Typical results are shown in Table II. Butylated hydroxytoluene (BHT) and hydroquinone (HQ) were also fed to determine whether these antioxidants would show similar toxicity in pregnant animals. Neither BHT nor HQ at the 0.3% level induced the prolonged gestation and mortality characteristic of DPPD. BHT at the 1.55% level caused a drastic weight loss in the pregnant females which was associated with fetal deaths. This response was not unexpected since at this level of feeding the rats were receiving each day about half the single dose LD_{50} of BHT as reported by Deichmann *et al.* (8).

The DPPD effect is more pronounced in animals fed the supplemented "stock diets" 10 days before insemination than in those fed the supplemented "vit. E-low diets fortified with *d*-α-tocopheryl acetate" starting on the day of insemination. Since these experiments were conducted at different times, comparisons should be made with caution. The most

TABLE II. Effect of DPPD* on Reproduction in Female Rats.

Group No.	Supplement and level in diet (%)	Fertility index	Litter efficiency	Mortality index	Mean length of gestation	Mean litter size	Viability index	Lactation index
Vit. E-low diet† + .001% *d*-α-tocopheryl acetate (supplemented diets fed starting on day of insemination)								
1	Negative control	14/17	100	0/17	22	7.7	73/ 85	71/73
2	DPPD .0125	8/11	60	1/11	23	6.2	18/ 31	18/18
3	” .0625	11/11	88	0/11	24	5.1	8/ 51	6/ 8
4	” .313	10/11	90	2/11	25	6.0	0/ 54	
5	” 1.55	8/11	50	1/11	26	7.3	0/ 29	
6	BHT .0125	9/11	100	0/11	22	8.7	71/ 78	70/71
7	” .0625	11/11	100	0/11	22	7.5	73/ 82	59/73
8	” .313	11/11	91	0/11	22	8.7	72/ 87	69/72
9	” 1.55	9/11	0	0/11	22			
Stock diet‡ (supplemented diets fed 10 days prior to insemination)								
1	Negative control	16/17	88	1/17	23	10.6	71/104	69/71
2	DPPD .0125	10/12	83	0/12	24	7.9	4/ 79	3/ 4
3	” .0625	15/17	70	5/17	25	4.9	0/ 49	
4	” .313	8/10	60	5/10	25	5.3	0/ 16	
5	” 1.55	11/13	33	7/13	25	4.7	0/ 14	
6	HQ .003	10/10	100	0/10	22	8.1	79/ 84	78/79
7	” .3	10/10	100	0/10	23	7.8	65/ 76	62/65

* Feed grade DPPD. † Diet #301 of Harris and Ludwig(9). ‡ Diet of Ames *et al.*(10) except with linseed meal replacing soybean meal and partially hydrogenated vegetable oil (Primex) replacing margarine oil.

sensitive criterion of DPPD feeding, a high incidence of early mortality of young, occurred in both experiments in groups fed 0.0125% DPPD, the lowest level tested. In another experiment, supplementation of the diet with 400,000 I.U./kg of vit. A or 0.1% of *d*-α-tocopheryl acetate failed to alleviate the toxicity. Those young rats which survived this early period of life (3 days) were able to suckle successfully, as evidenced by a normal lactation index. Considerable maternal mortality occurred at levels of 0.0625% DPPD and higher in the stock diet. The abnormality leading to death seemed to be largely mechanical. Gestation was prolonged, in some instances as much as 36%. Probably the fetuses continued to grow *in utero* until, when parturition did occur, the birth process was exceedingly difficult and prolonged. Many of the young were born dead and most of the mothers that died did so during parturition. Other symptoms occasionally observed were vaginal bleeding and prolapse of the uterus. The physiological defect caused by DPPD is probably failure of "the trigger mechanism" which starts the birth process at the proper time.

Summary and conclusions. 1) Vit. E. bioassays (prevention of resorption-gestation) of both methylene blue and DPPD indicated a low order of activity. However, this apparent tocopherol-like potency was due probably to a protective or sparing effect on small residual quantities of vit. E in the diet and in the body tissues. Under conditions where the diet was rigorously freed of tocopherol and the depletion period was extended, methylene blue and DPPD were found by other investigators to be completely devoid of vit. E activity. 2) At higher levels of supplementation, both methylene blue and DPPD were toxic. Methylene blue in doses of 100 mg/day killed pregnant female rats. Feeding pregnant rats diets containing DPPD at a level of 0.0125% in the diet resulted in mortality of their young. Higher levels of DPPD induced prolonged gestation and maternal mortality. Neither BHT (0.313%) nor hydroquinone (0.3%) produced these symptoms of toxicity, but BHT at the 1.55% level in the diet resulted in drastic loss of weight and fetal deaths.

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1. Mason, K. E., and Harris, P. L., *Biol. Symposia*, 1947, v12, 459.

2. Moore, T., Sharman, I. M., and Ward, R. J., *Proc. Nutrition Soc.*, 1953, v12, *v*.

3. Christensen, F., Dam, H., and Gortner, R. A., *Acta Physiol. Scandinavica*, 1956, v36, 87.

4. Dam, H., and Granados, H., *Acta Pharmacol. Toxicol.*, 1952, v8, 47.

5. Moore, T., *Experientia Supp.* 1, 1953, 210, Verlag Birkhausen, Basel.

6. Johnson, B. C., and Goodyear, S., Third Inter. Congr. on Vitamin E, 1955, abstract No. 103, 46.

7. Johnson, B. C., *Vitamina E, Atti del terzo Congresso Internazionale Venezia* 1955, 1956, 450, Edizioni Valdonega, Verona, Italy.

8. Deichmann, W. B., Clemmen, J. J., Rakoczy, R., and Bianchine, J., *A. M. A. Arch. Ind. Health*, 1955, v11, 93.

9. Harris, P. L., and Ludwig, M. I., *J. Biol. Chem.*, 1949, v179, 1111.

10. Ames, S. R., Ludwig, M. I., Swanson, W. J., and Harris, P. L., *J. Am. Oil Chemists Soc.*, 1956, v29, 151.

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Mouse Pathogenicity with Gastric Mucin as an Index of Staphylococcus Virulence. (22657)

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Bacterial virulence, defined broadly as the capacity of an organism to invade and multiply in tissues and produce disease or death, is best measured by observing pathological effects induced by the microorganism in a suitable host. Animal virulence tests, although employed for a number of other bacterial species, have not been applied to staphylococci chiefly because of their inconstant and irregular pathogenicity for laboratory animals. Such biochemical properties as coagulase, pigment and hemolysin production as well as mannite fermentation are utilized instead with the greatest reliance being placed on the first, but the validity of these criteria as indices of virulence in all instances has been subject to serious question by many investigators. Lack and Wailling(1), as one example, in a study on the factors contributing to pathogenicity of staphylococci, state, "While there is strong evidence that the absence of coagulase production is associated with nonpathogenicity, there is no evidence that all coagulase-producing strains are pathogenic."

Hog gastric mucin has found wide application as an adjuvant in converting infections caused by organisms considered of low virulence for mice on intraperitoneal injection into rapidly lethal ones. Among the numerous bacterial species reported amenable to its action in this regard are some strains of staphylococci(2-4). Ercoli *et al.*(5) have shown that staphylococci injected intraperitoneally into mice with mucin remain viable and spread from the abdominal cavity, while without it they are rapidly destroyed. It was believed of interest to ascertain whether the mouse could be converted from a relatively resistant host to staphylococcus infection by the intraperitoneal route into one readily susceptible, by means of the adjuvant, and if so, to determine whether this procedure could be adapted to the differentiation of virulent from avirulent members in the same manner that animal inoculations are employed with corynebacteria, clostridia, mycobacteria, etc. To facilitate this evaluation, the *in vitro* presumptive biochemical reactions of a series of strains of staphylococci were compared with their intraperitoneal pathogenicity for mice with mucin.

Materials and methods. 60 strains of staphylococci recently isolated from a variety of such clinical sources as suppurative lesions, blood, nose and throat, urine and skin were employed. 5% hog gastric mucin (Type 1701-Wilson) was prepared according to Miller(6). Ten albino Swiss mice, weighing approximately 15-20 g, were used for each bacterial strain, of which 5 received 0.5 ml of 5% hog gastric mucin and the remainder 0.5 ml of physiological saline intraperitoneally 15-20 minutes prior to the intraperitoneal injection into each mouse of 0.25 ml of a 16 hour veal infusion broth culture. Animals were observed 48 hours and all deaths were recorded. In addition, one group of 10 mice received 0.75 ml of mucin and another group of 10 mice received 0.75 ml of veal infusion broth intraperitoneally in order to test toxicity of mucin and broth alone without any fatalities being noted in either group.

Coagulase production was determined by tube test. Contents of an ampule of desiccated rabbit plasma (Difco) was suspended in 3 ml of distilled water and 2 drops of a 16 hour veal infusion broth culture of each strain under investigation was added to 0.5 ml of the plasma solution and incubated in a water bath at 37°C for 3 hours. A positive test was distinct clotting of the tube's contents. Presence of yellow to deep orange colonies after incubation at 37°C for 2 days and at room temperature near the light for 3 more days on a medium containing 2 parts 2% veal infusion agar and 1 part sterile homogenized milk indicated pigment production. Hemolysin production was manifest by distinct clear zones around bacterial colonies on 5% human blood agar plates within 72 hours at 37°C. Mannite fermentation was denoted by red colonies and the formation of a red color in the surrounding medium, after incubation at 37°C for 72 hours, on a veal infusion agar plate containing 1% mannite and Andrade's indicator.

Results. The significant enhancement in pathogenicity for mice achieved with the aid of gastric mucin with a considerable proportion of the bacterial strains studied is demonstrated in Table I. It may be noted from the table that whereas only 5 of the 60 strains were able to produce a mortality of at least 3 out of 5 mice without mucin, 42 proved lethal to the same degree with the adjuvant. Thus, by its use, the mouse was converted from a host resistant to staphylococcus infection via the intraperitoneal route into one readily susceptible with 37 strains. In no instance was any strain found pathogenic to mice without mucin and innocuous with it.

TABLE I. Enhancing Effect of Gastric Mucin upon Pathogenicity of 60 Strains of Staphylococci for Swiss Mice.

	Without mucin		With mucin	
Mouse mortality*	No. strains	% of total	No. strains	% of total
0/5	47	78.3	13	21.7
1/5	3	5.0	3	5.0
2/5	5	8.4	2	3.3
3/5	1	1.7	8	13.3
4/5	2	3.3	14	23.4
5/5	2	3.3	20	33.3

* Numerator = No. died. Denominator = Total No. animals inj.

To facilitate comparison between *in vitro* and animal tests, all strains fatal to at least 3 out of 5 mice with mucin were arbitrarily classified as pathogenic and hence virulent. Accordingly, 42 of the 60 strains investigated were found virulent and 18 non-virulent. The number and proportion of microbial strains found mouse pathogenic or non-pathogenic in relation to their reactions to each of the presumptive *in vitro* tests is summarized in Table II. Examination of Table II reveals that although mouse pathogenicity with mucin was not in complete accord with the results of the biochemical tests, a degree of correlation ranging from high to fair did exist between both, depending upon the test. Excepting hemolysin production and varying with each criterion, strains reacting positively were generally pathogenic to mice, while the negative reactors were non-pathogenic in the main. As for hemolysin production, 73.8% of the 42 hemolytic strains proved pathogenic, as were 61.1% of the 18 non-hemolytic strains. The relationship between coagulase production and virulence as determined by the procedure reported upon is of particular interest for it provides experimental corroboration for the observation of Lack and Wailling(1), quoted above. Thus only 1 of the 11 strains that failed to produce coagulase proved pathogenic. On the other hand, though 41 of the 49 coagulase positive strains were pathogenic, the remaining 8 were found non-lethal to the experimental animals.

In light of the questionable validity of the biochemical indices in some instances and in view of a number of discrepancies noted between the *in vitro* reactions and mouse patho-

TABLE II. Intraperitoneal Mouse Pathogenicity with Mucin of Staphylococci in Relation to Their *In Vitro* Reactions.

			Mouse pathogenicity with mucin			
			Pathogenic		Non-pathogenic	
In vitro test	Reaction	Total strains	No.	% total	No.	% total
Coagulase	Positive	49	41	83.7	8	16.3
	Negative	11	1	9.1	10	90.9
Pigmentation	Positive	39	34	87.2	5	12.8
	Negative	21	8	38.1	13	61.9
Hemolysin	Positive	42	31	73.8	11	26.2
	Negative	18	11	61.1	7	38.9
Mannite fermentation	Positive	52	41	78.9	11	21.1
	Negative	8	1	12.5	7	87.5

genicity with mucin, the animal virulence test reported upon is proposed as a supplementary method for directly assessing this property in staphylococci since it represents the determination of the capacity of a strain to induce death, which is the acme of virulence.

Summary. Significant enhancement of mouse pathogenicity was observed following intraperitoneal injection of staphylococci with gastric mucin. By means of the adjuvant, the mouse was converted from a host resistant to staphylococcus infection by this route into one readily susceptible with 37 strains. High to fair degree of correlation, depending upon the test, was found between the *in vitro* presumptive biochemical tests and intraperitoneal mouse pathogenicity with mucin but some discrepancies were noted between both. The procedure under consideration is proposed as a supplementary method for the direct determination of virulence of staphylococci.

1. Lack, C. H., and Wailling, D. G., *J. Path. and Bact.*, 1954, v68, 431.
2. Nungester, W. J., Wolff, A. A., and Jourdonais, L. F., PROC. SOC. EXP. BIOL. AND MED., 1932, v30, 120.
3. Anderson, C. G., and Oag, R. K., *Brit. J. Exp. Path.*, 1939, v20, 25.
4. Ercoli, N., Lewis, M. N., and Harker, E. M., *Am. J. Med. Sci.*, 1945, v209, 621.
5. ———, PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 273.
6. Miller, C. P., *ibid.*, 1935, v32, 1136.

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Comparison of Continuous and Strip Paper Electrophoresis Technics for Study of Serum Glycoproteins.* (22658)

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Paper strip electrophoretic technics are now widely used for fractionation studies of serum proteins. Application of the Hotchkiss-McManus technics for tissue polysaccharides to paper strips by Koiw and Gronwall(1) allows studies of carbohydrate components of the serum proteins. Technics for continuous paper electrophoresis apparatus using vertical curtains have been described by Grassmann and Hannig(2) and by Durrum(3). The use of such equipment allows the collection of serum fractions in quantities which make possible chemical analysis for the glycoprotein and protein components. The work described here was undertaken to compare results obtained by continuous electrophoresis technics with the less tedious paper strip methods.

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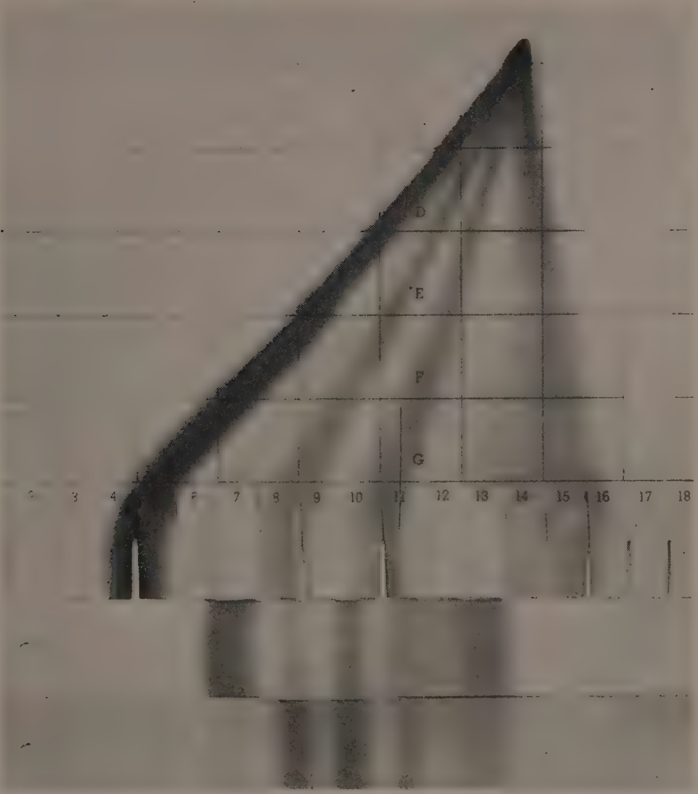

FIG. 1. Typical continuous and strip paper electrophoretic patterns. (Albumin is at the left in all patterns.) Top: Continuous electrophoretic curtain stained for proteins with bromophenol blue. Middle: Strip pattern stained with bromophenol blue. Bottom: Similar strip of same serum after development of periodic acid Schiff reaction for glycoproteins.

Methods. A Karler-Misco Continuous electrophoretic unit[†] was used with a constant voltage supply.[‡] The plate supporting the paper curtain was found to make too much contact with the paper and was modified by making a plastic plate with fewer contact points. A slow stream of air was bubbled through the anode chamber to prevent plating out of buffer ions. A .045 barbital buffer (pH 8.6) was used for all studies. The apparatus was allowed to equilibrate for about 8 hours until the current became stable at 12 milliamperes. Electrophoresis was carried out at 350 volts for 24-48 hours. A paper wick (S and S No. 470) was used to feed the serum sample to the paper curtain.

At the end of each run the curtain was removed and the pattern developed with bromophenol blue in the same manner as used for strip electrophoresis. The collecting tubes, previously calibrated at 3 ml, were labeled and the volume in each adjusted to 3 ml. Aliquots were taken from each tube for estimations of protein by the biuret reaction and bound carbohydrate by the method of Shetlar, Foster, and Everett(4). Samples were also taken from each tube for paper strip electrophoresis. From these studies the protein constituents of each tube were identified. If more than one fraction was noted on the paper strip, it was scanned in the Spinco Analy-

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[†] Microchemical Specialties Co., Berkeley, Calif.

[‡] Model 1400 Research Specialties Co., Berkeley, Calif.

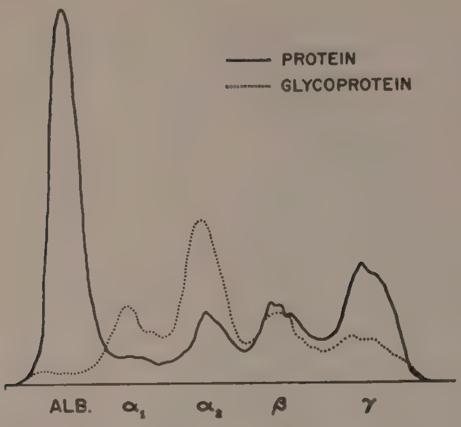

FIG. 2. Typical densitometer tracings of strip electrophoresis patterns for proteins and glycoproteins. Patient with active rheumatoid arthritis. Glycoprotein, 201 mg/100 ml; protein, 8.05 g/100 ml.

trol and the relative amounts of each fraction estimated.

Paper strip electrophoresis. Strip electrophoresis was carried out essentially by the method of Block, Durrum and Zweig(5) using Spinco hanging strip electrophoresis cells. 3MM filter paper was used for all work. Electrophoresis was conducted at constant current (5 milliamperes for 8 strips) using barbital buffer, .075M, pH 8.6. Duplicate strips, 10 λ for protein and 30 λ for glycoprotein studies, were made of each sample. For estimation of protein the strips were developed with bromophenol blue essentially as described by Block *et al.*(5). For glycoprotein studies, the strips were subjected to the periodic acid Schiff reaction as applied by Koiw and Gronwall(1) except that the reducing solution was prepared as described by Roboz *et al.*(6). The protein strips were quantitated with the Spinco Analytrol using blue filters; the glycoprotein strips were similarly evaluated with the same instrument using Klett 52 (green) filters. The total area under the protein curve was equated to the serum protein as determined by the biuret reaction, and the area under the glycoprotein curve was similarly made equal to the total serum glycoprotein obtained by the tryptophan method(4).

Results. Typical electrophoretic patterns by both technics are shown in Fig. 1, and typical analytrol curves for protein and glycoprotein are presented in Fig. 2. The difference in distribution of protein and bound carbohydrate is illustrated in both figures.

Average glycoprotein contents of serum fractions of patients with cancer and arthritis and for normal and pregnant subjects are summarized in Table I. The corresponding protein values are tabulated in Table II. Even with the small number studied, differences are apparent between the 4 groups.

In order to obtain some measure of the agreement between the two methods, the correlation coefficients for the 2 methods for the glycoprotein and protein of each fraction were calculated. Correlation coefficients for the glycoprotein moieties were: Albumin, .74; α_1, .81; α_2, .71; β, .78; and γ-globulin, .90; for the protein components they were: Albumin, .84; α_1, .75; α_2, .79; β, .55; and γ-globulin, .93. With the exception of β-globulin protein all of these correlation coefficients are significantly different from O at the 1% level of probability.

Discussion. Two different electrophoretic methods have been compared in this study. Not only do the electrophoretic technics vary, but the reactions used to quantitate the results are also different. For the continuous technic the protein of each fraction was estimated by the biuret reaction, while proteins by the strip method were estimated by densitometer readings made directly on the bromophenol blue dyed strip. Many studies have been made of the bromophenol blue staining technic as applied to paper strip electrophoresis(5) and a multiplicity of factors which affect the quantitation of protein fractions by this method have been noted. At present, it appears the best approach to use of this technic for study of serum fractions is an arbitrary one with standardized dyeing and scanning procedures. The continuous paper electrophoresis technic would appear to be more direct in its approach; however, this method is more difficult and is consequently unlikely to gain favor for extensive studies. The agreement of the continuous with the strip method

TABLE I. Distribution of Glycoprotein in Different Conditions.

Condition	No.		Bound carbohydrate* associated with Albumin	α_1	α_2	β	γ	Total†	Seromucoid‡
Cancer	5	Strip	18	48	72	32	18	188	41
		Continuous	20	49	67	31	20		
Rheumatoid arthritis	2	Strip	15	38	80	35	30	198	27
		Continuous	18	30	79	35	38		
Late pregnancy	2	Strip	12	22	49	44	25	152	17
		Continuous	11	25	54	39	22		
Normal	3	Strip	12	18	36	33	24	122	15
		Continuous	13	16	35	31	24		

* mg/100 ml of serum.

† Bound hexose polysaccharide by tryptophan method(4).

‡ Determined by tryptophan method after isolation by method of Weimer *et al.*(7).

TABLE II. Comparison of Protein Distribution.

Condition	No.		Protein* associated with Albumin	α_1	α_2	β	γ
Cancer	5	Strip	39.6	10.0	16.7	14.9	18.8
		Continuous	39.9	8.9	16.6	13.9	20.7
Rheumatoid arthritis	2	Strip	43.4	5.0	11.6	14.2	25.8
		Continuous	42.9	6.0	12.5	11.4	27.2
Late pregnancy	2	Strip	46.8	7.4	12.6	18.4	14.8
		Continuous	43.6	7.6	16.1	15.9	16.8
Normal	3	Strip	53.6	5.8	9.1	13.6	17.9
		Continuous	58.1	3.9	8.0	10.8	19.2

* Expressed as a % of total serum protein.

tends to strengthen the use of the latter method for routine work.

The agreement between glycoproteins by the two technics is rather surprising considering the great difference in technics and quantitation. The periodic acid Schiff reaction has not been evaluated as a quantitative test for serum glycoproteins, and the tryptophan method of Shetlar *et al.*(4) has not been thoroughly studied for purified serum fractions. However, the agreement noted in this paper tends to strengthen both of these technics as tools for investigations of glycoproteins.

Summary. A continuous paper electrophoretic technic was compared with a paper strip electrophoretic technic as a tool for the study of serum protein and glycoprotein fractions. Protein and glycoprotein for the continuous electrophoretic study were quantitated by analyses of the fractions by the biuret and tryptophan reactions. The glycoproteins by the strip technic were quantitated by the periodic acid Schiff reaction and the protein strips by the bromophenol blue staining. In both cases the strips were evaluated with an automatic photoelectric scanner. Good agreement between the two methods for both protein and glycoprotein was noted.

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1. Koiw, E., and Gronwall, A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 244.

2. Grassmann, W., and Hannig, K., *Naturwissenschaften*, 1952, v37, 397.

3. Durrum, E. L., *J. Am. Chem. Soc.*, 1951, v73, 4875.

4. Shetlar, M. R., Foster, J. V., and Everett, M. R., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 507.

5. Block, R. J., Durrum, E. L., and Zweig, G., 1955, *Paper Chromatography and Paper Electrophoresis*, Part II, Academic Press, Inc., New York.

6. Roboz, E., Hess, W. C., and Forster, F. M., *A. M. A. Arch. Neurol. and Psychiat.*, 1955, v73, 563.

7. Weimer, H. E., and Moshin, J. R., *Amer. Rev. Tuberc.*, 1953, v68, 594.

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Characteristics of the New Respiratory Viruses (Adenoviruses[*]) II. Stability to Temperature and pH Alterations.[†] (22659)

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To investigate the biologic characteristics of the respiratory viruses isolated independently by Rowe and co-workers(1) and by Hilleman and Werner(2) it was important to determine the stability of these agents under a variety of conditions. For practical considerations it was desirable to investigate the experimental conditions under which these viruses were inactivated, as well as the optimum and most practical conditions for handling and storing the agents. It is the purpose of this paper to present evidence that the 4 prototype viruses studied are relatively stable under varying conditions of pH and temperature.

Materials and methods. Tissue culture. Strain HeLa cells (Gey) derived from an epidermoid carcinoma of the uterus were employed(3). The methods used for the serial propagation of these cells and preparation of tube cultures were identical to those previously described(4,5). The nutrient fluid consisted of 40% human serum and 60% Hanks' balanced salt solution(3). *Viruses.* The viruses employed were types 1, 2, 3 and 4 of the new respiratory viruses(1,2) originally termed the Adenoid Degeneration or "AD" agents(1) or the RI-67 agents(2). More recently Huebner *et al.* have suggested the name adenoidal-pharyngeal-conjunctival (APC) viruses for this group of agents(6). A committee composed of interested investigators recently suggested the designation "Adenoviruses" for this group of agents(7), and this terminology will be employed hereafter. Types 1, 2, and 3 were kindly supplied by Dr. R. J. Huebner and type 4 (also termed RI-67(2) or ARD virus(4)) was furnished by Dr. M. R. Hilleman. The type designations are the same as those suggested by Huebner and co-workers (6). The viruses were propagated in HeLa cells using a maintenance mixture consisting of Scherer's MS 90%(3), and chicken serum 10% or MS 67.5%, chicken serum 7.5% and tryptose phosphate broth (Difco) 25%(5). Virus pools were prepared by freezing and thawing infected cells as previously described (5). Viruses were stored in sealed glass ampules at —70°C. *Infectivity titrations.* Serial 1:3.2 ($10^{-0.5}$) dilutions were prepared in Hanks' balanced salt solution, and 0.1 ml of each dilution was inoculated into each of 2 HeLa cell culture tubes. Inoculated tubes were incubated at 36°C for 6 days. A culture was considered infected when 50% or more of the HeLa cells had undergone specific cytopathogenic changes. The infectivity titer end-point was calculated as the highest dilution of virus which infected one-half of the tubes inoculated. *Alterations of pH.* To adjust the viral suspensions to the desired pH, 0.5 N HCl or NaOH was employed. The quantity of HCl or NaOH required to obtain a desired pH was preliminarily determined using uninfected maintenance mixture. By this relatively crude method the number of drops of HCl or NaOH delivered by a calibrated capillary pipette in order to obtain a desired pH was placed in appropriate rubber stoppered tubes. To each tube was added 1.0 ml of virus infected maintenance mixture. After at least 5 minutes to permit complete mixture, the pH of each solution was determined electrophotometrically. The viral suspensions adjusted to the desired hydrogen ion concentrations were allowed to stand at room temperature for 30 minutes, after which the pH

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[*] Name suggested to designate group of viruses previously termed AD, APC, or RI viruses(7).

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TABLE I. Storage of Adenoviruses at 4°C.

Virus	Virus infectivity titer after storage at 4°C (days)			
	None	18	44	70
	(log)			
Type 1	-2.75	-2.75	-2.5	-2.75
2	-2.25	-2.5	-2.25	-2.25
3	-2.75	-3.0	-2.5	-2.75
4	-2.25	-1.75	-2.0	-2.0

TABLE II. Stability of Adenoviruses at Room Temperature (22-23°C).

Virus	Infectivity titer after storage at 22-23°C (days)					
	None	2	4	7	10	14
	(log)					
Type 1	-3.5	-3.5	-3.5	-3.5	-3.5	*
2	-3.0	-3.5	-3.0	-3.0	-3.0	-2.5
3	-3.0	-3.0	-3.0	-3.0	-3.0	-3.5
4	-3.5	-3.5	-3.5	-3.5	-3.5	*

* Not tested.

of each was rapidly adjusted to pH 7.4-7.6 with 0.5 N HCl or NaOH. The total volume of NaOH and HCl which had been added to each tube had been recorded so that all viral samples, including the unaltered controls, were finally adjusted to the same volume with maintenance mixture. To determine viral inactivation, infectivity titrations were carried out on each aliquot in a given experiment at the same time. For one group of experiments a 0.1 M phosphate buffer at pH 6.5 was used to adjust the pH of the viral suspensions.

Results. Stability on storage. The 4 prototype viruses studied have been stored in the frozen state in glass sealed ampules at —70°C in a cabinet refrigerated with solid CO_2 and at —30°C in a mechanically refrigerated box for 19 and 7 months, respectively, the longest periods tested, without detectable decrease in infectivity titers.

It was next of considerable importance to determine whether the property of infectivity was stable at 4°C, ordinary refrigerator temperature. Pools of virus infected tissue culture homogenate were placed in screw-topped tubes at 4°C, and after 18, 44, and 70 days aliquots were removed and stored at —30°C until the completion of the test period. Infectivity titrations were done on all samples at the same time. The results of this experiment, summarized in Table I, indicate that these viruses can be stored at 4°C for over 2 months without loss of infectivity titer.

A similar experiment was carried out to determine whether the adenoviruses were inactivated when held at room temperature (from 22°-24°C) during the experimental period. The data from this experiment, presented in Table II, indicate that these viruses retained their infectivity titers at room temperature for 10 to 14 days.

Stability at 36°C. The above data indicated that the adenoviruses are relatively stable when stored over a wide range in temperature. The rate of inactivation of these agents when incubated at the temperature utilized for propagation of the viruses, *i.e.*, 36°C, was next determined. The results are summarized in Table III. For these experiments it was necessary to buffer the type 4 virus suspension in order to maintain a pH of 7.4. Preliminary experiments had shown that if the pH of type 4 virus preparations increased above 7.8 during incubation, the infectivity titer began to decrease within 24 to 48 hours; by 4 or 5 days the preparation would usually be non-infectious. As noted in Table III, however, when the pH of type 4 virus suspensions was maintained at a constant pH of 7.4 during incubation, this agent

TABLE III. Stability of Adenoviruses at 36°C.

Virus	Infectivity titer after incubation at 36°C (days)							
	None	1	2	3	4	5	6	7
	(log)							
Type 1	-4.75	-4.75	-4.75	-4.0	-4.5	-4.5	-4.25 or >*	-4.25 or >*
2	-3.5	-3.75	-3.75	-3.5	-3.75	-3.25	-3.75	-3.5
3	-3.5	-3.25	-3.0	-3.25	-3.0	-3.0	-3.0	-3.0
4†	-3.25	-3.0	-3.0	-3.0	-3.0	-3.0	-2.5	-2.5

* Highest dilution tested, $10^{-4.0}$, infected both tubes.
† Buffered at pH 7.4 with 0.01 M phosphate buffer.

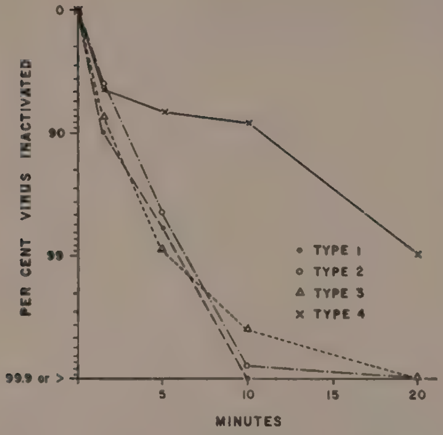

FIG. 1. Inactivation of adenoviruses at 50°C. Five 1.0 ml aliquots of each virus in tightly stoppered tubes were incubated in a 50°C water bath. At indicated intervals an aliquot of each virus was removed from the 50°C water bath and immediately immersed in an ice-water bath at 1°C. Infectivity titrations on all samples were done on the same day. Each endpoint represents geometric mean of 2 experiments.

was as stable as the other types. These data give further evidence of the marked stability of these agents, for when they were incubated at 36°C for as long as 7 days there was no measurable decrease of the infectivity titers by the methods employed.

Inactivation at 50°C and 56°C. To determine the rate and kinetics of the inactivation of the adenoviruses, the 4 agents under study were heated in 1.0 ml volumes at 50°C and 56°C for increasing periods. At the termination of the predetermined period of incubation the heated aliquot was immediately immersed in an ice-water bath at 1°C. Infectivity titrations on all samples of a single virus were done on the same day with a single lot of HeLa cell tubes. The results of 2 experiments with each virus are summarized in Fig. 1. Types 1, 2, and 3 viruses were inactivated at similar but not identical rates, whereas the type 4 virus was more resistant to inactivation at 50°C. It is of interest to note that the curves of inactivation of types 1 and 2 viruses could be interpreted to be straight line reactions. On the other hand, the curves of inactivation for types 3 and 4 viruses are not straight lines, which indicates that these agents were not inactivated at a uniform rate during successive unit time intervals at 50°C. No such differences in the rates of inactivation of these agents at 56°C were detectable, as all 4 viruses were more than 99.9% inactivated when incubated for only 2½ minutes and no residual infectivity could be detected after 5 minutes incubation at this temperature. That types 1-3 are non-infectious after incubation at 56°C for 30 minutes has been reported(6).

Degree of viral inactivation by varying pH. Multiplication of adenoviruses in HeLa cell cultures and the cytopathogenic alteration of the host cells induced by these agents is accompanied by an increase of lactic acid in the culture fluids(6,8,9) which may reduce the pH of the culture as low as pH 6.5 to 6.7(10). To determine whether the viruses may be inactivated during incubation of viral infected cultures the stability of adenoviruses was tested when the viral suspensions were adjusted to pH 6.5 and incubated at 36°C. These experiments clearly demonstrated that types 1-4 adenoviruses were not inactivated when incubated at 36°C for 24 hours at a pH of 6.5, conditions to which the viruses are often exposed during infection of HeLa cultures. To determine the stability of these agents under conditions which might be employed for chemical purification of viruses, as well as for other practical purposes, the inactivation of these agents over a wide pH range was also studied. The cumulative results of 2 or 3 experiments with each agent are summarized in Fig. 2. These data indicate that the adenoviruses are relatively stable at 22°-23°C between pH 6.0 and 10.0. It is of interest that type 4 virus was slightly less stable at acid pH's and type 3 virus was inactivated to a greater extent between pH 9 and 10 than were the other 3 prototype viruses studied. That these agents were not inactivated to a greater extent at pH's as low as 1.5-3.0 indicates that the infectious property is very stable under these unphysiologic conditions. Except for type 3 virus the agents under study were not inactivated to a significant degree between pH 7 and 10. At higher pH's, however, the viruses were readily inactivated.

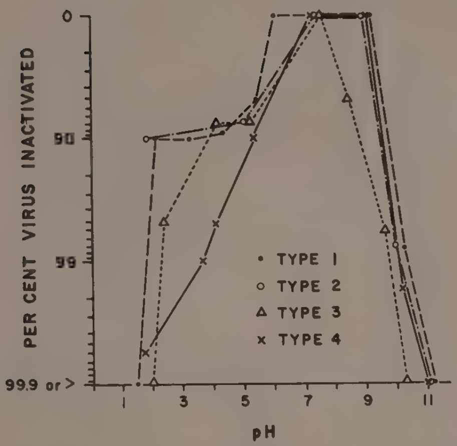

FIG. 2. Inactivation of adenoviruses by acid and alkaline pH changes. Adjustments of pH were made with 0.5 M NaOH or HCl. After pH change, all samples were held at 22-23°C for 30 min. pH of each aliquot was then adjusted to pH 7.4-7.6. Infectivity titrations on all aliquots in a single experiment were done on the same day. Each end-point represents geometric mean of results from 2 or 3 experiments.

and at pH 11.0 infectious virus was not detected with any of the 4 types tested.

Discussion. The data presented indicate that the prototype viruses of types 1-4 of the new respiratory viruses(1,2), adenoviruses (7), are relatively resistant to inactivation both by heat and by pH changes. It must be emphasized, however, that only a single viral strain of each type was investigated; the variations of these characteristics within strains of a single type were not determined. Comparison of the types 1-4 viruses with the stability of other animal viruses indicates that their stability to heat and pH alterations is of the same order as the poliomyelitis(11-14) and Coxsackie viruses(15,16). All of these agents resist inactivation by ether(6,17-20). Further comparisons point out that the adenoviruses are considerably more stable to temperatures from 4°C to 50°C and pH alterations than are the influenza(21,22,23), mumps, and Newcastle disease viruses(24, 25). The stability of the adenoviruses when they are maintained at 4°C, room temperature (22°-23°C), or in the frozen state (—30°C to —70°C) facilitates attempts to isolate these agents from human sources. Furthermore, the capacity of these viruses to resist inactivation upon incubation at 36°-37°C eliminates a constant source of error from studies of the characteristics of the multiplication cycles(9,10) as well as other host-virus reactions(9,10).

The ecology of an infectious agent in many ways must be a reflection of the characteristics of the agent. That a virus can remain without inactivation of its important property of infectivity under a wide range of conditions should permit it to persist in nature either within or outside its host. These characteristics of stability similarly should permit the agents to spread from host to host under conditions which otherwise would make contagion difficult. Indeed, it may be considered that a basic understanding of the biologic and bio-physical characteristics of a virus not only facilitates insight into the activity of the agent in nature, but also permits hypotheses to be developed concerning the basic nature of the viral particle.

Summary. Types 1-4 of the new respiratory viruses, termed adenoviruses, were relatively stable when exposed to temperatures ranging from 4°C to 36°C. Type 1-3 viruses were inactivated in 10 to 20 minutes at 50°C, but the infectivity titer of type 4 virus was reduced less than 2 log units (99%) in 20 minutes. The adenoviruses were also resistant to inactivation at pH 6.0 to 9.0. The patterns of inactivation of the 4 viruses by alterations of pH as well as incubation at 50°C were similar, although consistent and significant differences among the 4 types were measured.

The excellent technical assistance of Miss Mary K. Dixon is gratefully acknowledged.

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1. Rowe, W. P., Huebner, R. J., Gilmore, R. K., Parrott, R. H., and Ward, T. G., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 570.

2. Hilleman, M. R., and Werner, J. H., *ibid.*, 1954, v85, 183.

3. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, v97, 695.

4. Ginsberg, H. S., Badger, G. F., Dingle, J. H., Jordan, W. S., Jr., and Katz, S., *J. Clin. Invest.*, 1955, v34, 820.

5. Ginsberg, H. S., Gold, E., and Jordan, W. S., Jr., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 66.

6. Huebner, R. J., Rowe, W. P., Ward, T. G., Parrott, R. H., and Bell, J. A., *New Eng. J. Med.*, 1954, v251, 1077.

7. Enders, J. F., Bell, J. A., and Dingle, J. H., Francis, T., Jr., Hilleman, M. R., Huebner, R. J., and Payne, A. M. N., *Science*, in press.

8. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G., and Parrott, R. H., *Am. J. Hyg.*, 1955, v61, 197.

9. Ginsberg, H. S., and Gold, E., *Fed. Proc.*, 1955, v14, 464. (Abst.)

10. Ginsberg, H. S., *Trans. N. Y. Acad. Sci.*, in press.

11. Kaplan, A. S. and Melnick, J. L., *Am. J. Pub. Health*, 1952, v42, 525.

12. Youngner, J. S., *Fed. Proc.*, 1956, v15, 624. (Abst.)

13. Loring, H. S., and Schwerdt, C. E., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 173.

14. Faber, H. K., and Dong, L., *ibid.*, 1946, v63, 575.

15. Melnick, J. L., and Ledinko, N., *J. Exp. Med.*, 1950, v92, 463.

16. Robinson, L. K., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 580.

17. Brodie, M., *J. Immunol.*, 1935, v28, 1.

18. Sulkin, S. E., and Zarafonetis, C., *J. Exp. Med.*, 1947, v85, 559.

19. Melnick, J. L., *Bull. N. Y. Acad. Med.*, 1950, v26, 342.

20. Sulkin, S. E., and Wallis, C. H., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 151.

21. Lauffer, M. A., Carnelly, H. L., and MacDonald, E., *Arch. Biochem.*, 1948, v16, 321.

22. Horsfall, F. L., Jr., *J. Exp. Med.*, 1954, v100, 135.

23. ———, *ibid.*, 1955, v102, 441.

24. Weil, M. L., Beard, D., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1948, v60, 561.

25. Horsfall, F. L., Jr., *Science*, 1956, v123, 674. (Abst.)

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Effect of Reduced Liver and Kidney Catalase Concentrations on Lethality of X Irradiation in Rats.* (22660)

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Hydrogen peroxide, which is toxic to mammals(1), is a product of the X irradiation of water(2). The high percentage of water in the body, coupled with the presence of the hydrogen peroxide-reducing enzyme, catalase, in most living organisms(3) suggests a possible interrelation of X irradiation, hydrogen peroxide, and catalase.

According to Feinstein *et al.*(4), about 75% of the total body catalase in Sprague-Dawley rats is present in the liver and another 7% in the kidneys. Heim *et al.*(5) have reported that a single injection of 1 g of 3-amino-1,2,4-triazole/kg of body weight in rats reduces the liver and kidney catalase concentration to about 11% of normal in several hours and that it takes more than 72 hr for the catalase to return to normal levels. It seemed of interest to determine whether reducing the liver and kidney catalase to low levels with this compound would increase the number of deaths in X-irradiated rats.

Materials and methods. Sprague-Dawley rats were used throughout. The 3-amino-1, 2,4-triazole (AT)[†] was used in concentrations of 50 mg/ml in water. Physiological saline (0.9%) was injected into all control animals. Both solutions were administered intraperitoneally in volumes of 20 ml/kg of body weight 4½ hr before either catalase assays or X irradiation. The results of the X-radiation experiments are shown in Table I. Inasmuch as the catalase-reducing effects of AT were reported for a different strain from that to those used in the experiments reported herein, the finding was checked. The liver catalase was assayed by the perborate method(6) and found to be depressed to 15-40% of that in

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* Work performed under USAEC Contract No. W-7405-eng-26.

[†] Generously supplied by the American Cyanamid Co.

TABLE I. Reduced Liver Catalase and Sensitivity to X Radiation (934 r).

Inj. material and amt inj. per kg rat wt	No. of rats inj. and wt range, g	No. of rats dying during 30-day observation period	Post-irradiation survival time, days
	Exp. I*		
AT, 1 g	12, 359–412	3	11 15 17
Saline (0.9%), 20 ml	12, 360–415	4	11 11 12 13
	Exp. II†		
AT, 1 g	20, 410–560	3	11 7 10
Saline (0.9%), 20 ml	20, 400–570	3	7 8 8

* Dose rate, 55.6 r/min.; target-rat distance, 89 cm.

† Dose rate, 51.4 r/min.; target-rat distance, 105 cm.

the controls.

The X-ray source was a General Electric Maxitron 250 operated at 250 kvp and 30 ma with a 3-mm Al filter and inherent filtration equivalent to 0.1 mm of Al (beryllium window). The hvl was 0.55 mm of Cu. A rotating circular lucite cage‡ (outside radius 17.5 cm) with 8 compartments was used as an exposure box. The cage was essentially the same in design as the circular mouse cage described by Upton *et al.*(7). Calibrations were made with a Victoreen 100-r condenser type ionization chamber, the chamber resting on a support in the cage and the thimble in the position of the center of the rat. The dose in both radiation experiments was 934 r; dose rates and vertical distances from the Victoreen to the level of the X-ray target were slightly different and are indicated in Table I.

Results. The catalase concentration was reduced to about 3% of normal 4½ hr after injection of AT, confirming a similar finding by Heim *et al.*(5) for Long-Evans rats. A check on the reduction of kidney catalase and the long recovery period for liver and kidney catalase also reported by these investigators does not seem necessary. The average value for liver catalase in the saline-injected control rats, 0.89 catalase unit, is very close to the 0.81 previously reported for the same strain (6).

Table I shows mortality data for the X-irradiated rats. In the first experiment, the radiation dose of 934 r resulted in a 30-day LD_{25} for the AT-injected animals and a 30-day LD_{33} for the saline controls. In the second experiment, the same level of radiation gave a 30-day LD_{15} for both groups. The data indicate that a low liver catalase level at the time of irradation does not increase the lethality of X radiation. Any possible role of the blood or blood-producing tissue catalase as a radiation protection agent is not elucidated by these experiments since AT does not reduce the blood catalase level(5) and since the catalase level in hematopoietic tissue was not measured. However, it seems clear that individual and strain differences in the lethal effects of X irradiation cannot be attributed to differences in liver and kidney catalase levels. It should be pointed out that the role of liver and kidney damage in 30-day irradiation mortality is not clear.

Summary. Liver and kidney catalase was markedly reduced in rats by the injection of 3-amino-1,2,4-triazole. X irradiation of these animals did not cause more deaths than in saline-injected control groups. It was concluded that differences in liver and kidney catalase levels cannot account for differences in sensitivity to the lethal effects of X irradiation.

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1. Feinstein, R. N., Cotter, G. J., and Hampton, M. M., *Am. J. Physiol.*, 1954, v177, 156.

2. Fricke, H., *Cold Spring Harbor Symp. Quant. Biol.*, 1935, v3, 55.

3. Sumner, J. B., and Somers, G. F., *Chemistry and Methods of Enzymes*, 2nd Ed., 1947, Academic Press, New York, p207.

4. Feinstein, R. N., Hampton, M., and Cotter, G. J., *Enzymologia*, 1953, v16, 219.

5. Heim, W. G., Appleman, D., and Pyfrom, H. T., *Science*, 1955, v122, 693.

6. Feinstein, R. N., *J. Biol. Chem.*, 1949, v180, 1197.

7. Upton, A. C., Conte, F. P., Hurst, G. S., and Mills, W. A., *Radiation Res.*, 1956, v4, 117.

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‡ Kindly loaned by Dr. A. C. Upton.

Specific Determination of Proline in Biological Materials.* (22661)

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Paper chromatographic studies on the amino acid metabolism of animal tissues cultivated *in vitro*(1) have shown that marked changes in the amino acid content of synthetic medium M 150(2,3) occur during the cultivation period. Medium M 150 contains 60 ingredients, including 21 amino acids, in a modified Tyrode's solution(4). Its complexity made it difficult to detect changes in certain amino acids, such as proline, that were present in low concentration or that reacted weakly with ninhydrin. Application of the isatin method for proline(5), or various modifications of this method(6-10), proved unsatisfactory since many other amino acids produced colors similar to that of proline. In earlier studies from this Laboratory(11-13), it was shown that the conventional ninhydrin procedure could be made specific for certain amino acids by treatment of the chromatograms with dilute acid or alkali subsequent to the ninhydrin development. Accordingly, similar procedures were tried with the isatin method and from these experiments, a specific and quantitative method for proline has been developed.

Methods. Samples of media for analysis (5.0 ml) were concentrated to dryness *in vacuo* over H_2SO_4 reconstituted in 0.2 ml of deionized water, and 10 λ portions used, without desalting. One-dimensional descending paper chromatograms were developed for 18 hours in either *n*-butanol-acetic acid-water or *n*-butanol-ethanol-water solvent systems. The chromatograms were then dried at 110°C for 2 to 3 minutes, dipped in isatin reagent (0.4% isatin dissolved in *n*-butanol containing 4% acetic acid), and reheated at 110°C for 10 to 15 minutes. Subsequent treatment of the chromatograms, with acid, to produce a selective color for proline is discussed in the succeeding paragraph. Full details of the general chromatographic procedures have been reported(1,11-13). Synthetic medium M 150 was prepared as described earlier(2,3). All chemicals and reagents were of the highest purity obtainable. Spectrophotometric measurements were made by cutting appropriate areas from the chromatograms and fixing them to the inner walls of 1 cm Corex cells filled with water. Optical densities were then determined in a Beckmann, Model DU. Direct measurements of the color intensity on the chromatograms were made with a densitometer (Welch Densichron).

Results. Selective development of color with proline and isatin. Paper chromatograms of medium M 150 were prepared, developed as described, and dipped in the isatin reagent. These chromatograms were then treated with various combinations of the acid and alkali procedures previously found suitable for specific color development with other amino acids(11-13). These experiments showed that washing the isatin-treated chromatograms with 1 N HCl, and suspending them at room temperature for 10 to 15 minutes intensified the blue proline chromogen, and at the same time caused almost complete fading of all other colored spots. Further

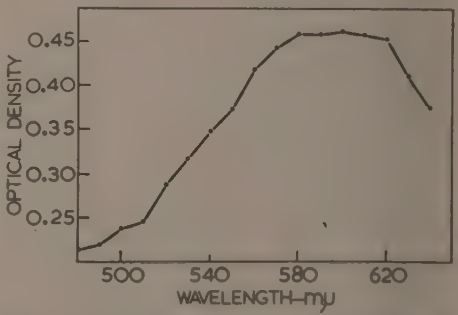

FIG. 1. Absorption curve of proline chromogen. Measurements made in Beckmann, Model DU, using 1 cm Corex cells containing area cut from developed chromatogram. 8 μg proline.

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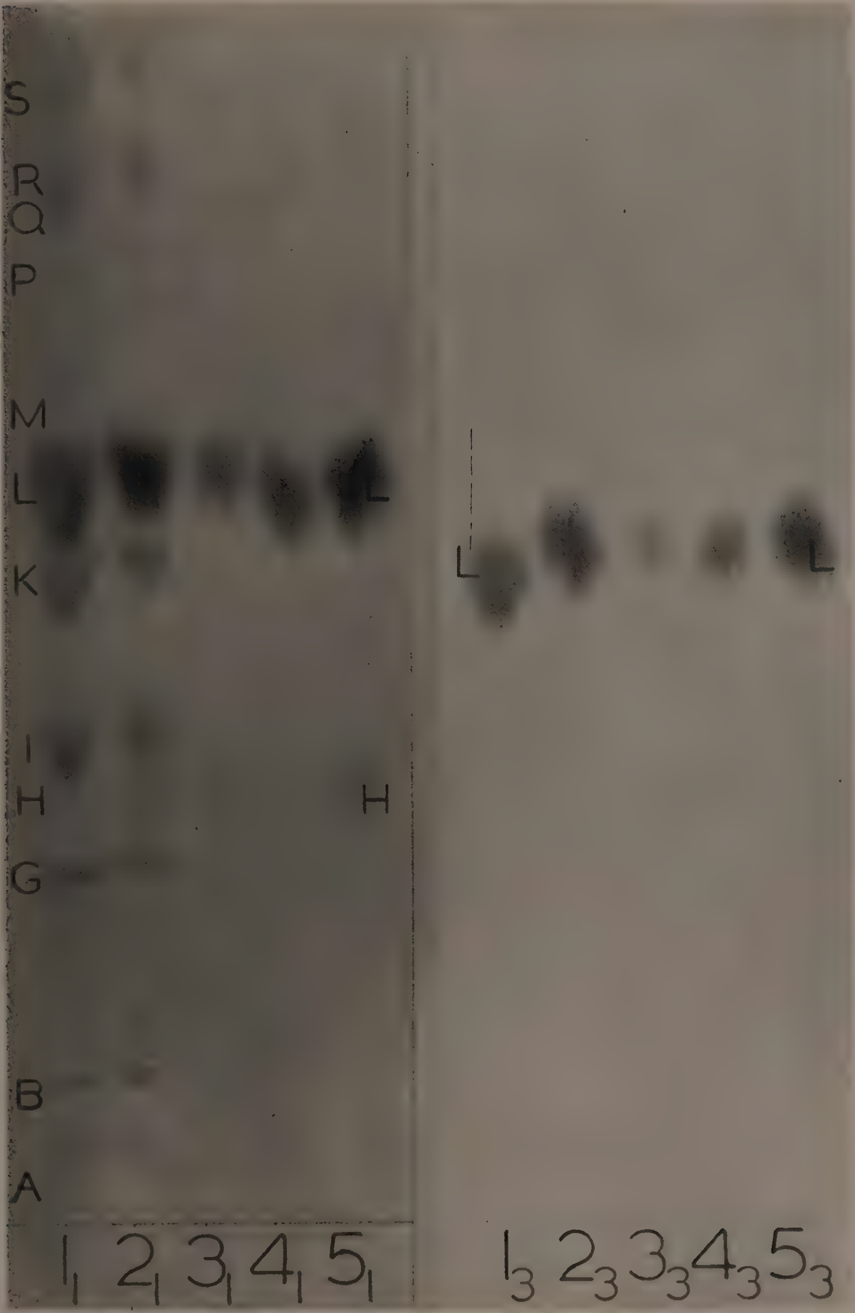
* The cooperation of Mr. C. E. Kerr, of this Department, in making the photographic preparations, is gratefully acknowledged.


S
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1_1 2_1 3_1 4_1 5_1
1_3 2_3 3_3 4_3 5_3

FIG. 2. Duplicate chromatograms, both developed by isatin, but chromatogram on right subsequently dipped into 1 N HCl and washed with water. 1_1 and 1_3, synthetic medium M 150, containing 20 μg of proline; 2_1 and 2_3, M 150 containing 25 μg of proline; 3_1 and 3_3, 5 μg of proline in water; 4_1 and 4_3, 10 μg of proline in water; 5_1 and 5_3, 25 μg of proline in water.

experiments established that this acid treatment bound the proline color so firmly to the paper chromatograms that the excess isatin reagent could be removed without eluting the proline chromogen. This was accomplished by washing with distilled water while the chromatograms were still damp. In this way, clear and distinct blue proline spots were obtained on a perfectly white background, free from any contamination with other amino acids. The absorption curve of this proline color is presented in Fig. 1 and shows a maximum at 600 mμ, with a broad plateau between 580 and 620 mμ. The absorption curve of the proline and isatin chromogen does not appear to have been reported previously.

Application of specific proline method to complex synthetic media. The application of this method to synthetic medium M 150 containing different levels of proline and hydroxyproline is illustrated in Fig. 2, showing 2 identical chromatograms, both developed by the conventional isatin procedure(5,6), but the second subsequently dipped into 1 N HCl and washed with water until the orange-yellow background color of the isatin reagent has been removed. By the conventional procedure (Fig. 2, 1_1 and 2_1), the presence of 20 and 25 μg of proline can be seen (L region), but interpretation of the chromatogram is made difficult by the large number of other amino acids also showing color development with isatin, and by overlapping of the proline spot with alanine (K) and tyrosine (M). Application of the new procedure (Fig. 2, 1_3 and 2_3) removes the interfering amino acids and proline is left as the only spot. The comparative behaviour of proline (L region) and hydroxyproline (H region) is also shown on these chromatograms. By the conventional isatin procedure (Fig. 2, 3_1 to 5_1), 5, 10, and 25 μg of proline show distinct color development, while the same quantities of hydroxyproline show only slight color formation. Application of the new procedure (Fig. 2, 3_3 to 5_3) clarifies the proline color development and, at the same time, completely removes the hydroxyproline color.

Quantitative measurement of proline by the new procedure. The gradation in color intensity with graded concentrations of proline (Fig. 2, 3_3 to 5_3) suggested that this procedure might be made quantitative. Attempts to elute the proline color with water, dilute HCl, dilute NaOH, methanol, ethanol, butanol, propanol, acetone, carbon tetrachloride, or benzene were unavailing, even though the extractions were performed with mechanical shaking until the filter paper was reduced to a pulp. Attempts were then made to determine proline directly on the chromatograms, employing a densitometer to measure peak density of graded levels of this amino acid. Following these measurements, the proline areas were cut from the chromatograms, placed on the inner walls of 1 cm absorption cells containing water, and the color intensity measured in a spectrophotometer at 600 mμ, employing as a blank a cuvette containing untreated filter paper. By careful placement of the proline spot to cover the center of the cuvette face, accurate measurements of peak density were obtained. The standard curves obtained by these two methods, based on the average results from 10 separate chromatograms containing graded amounts of proline, are presented in Fig. 3. By the spectrophotometer method (Fig. 3, Curve A), a linear response is exhibited over the concentration range of 1 to 16 μg. With the densitometer

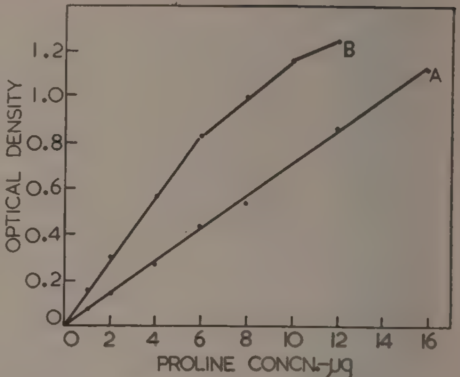

FIG. 3. Standard curves for graded concentrations of proline in M 150. Curve A, measurements made in Beckmann at 600 mμ. Curve B, measurements made on chromatograms with a densitometer. All points represent avg values from 10 separate chromatograms containing total amounts of proline specified.

method (Fig. 3, Curve B), linearity is maintained from 1 to 6 μg, then falls off. Attempts to maintain linearity by measuring both area and peak density were unsuccessful, and, for this reason, the spectrophotometer method is recommended for use.

Discussion. The isatin method has been used for determination of proline in known amino acid mixtures(5-10) but application of this method to complex biological media is difficult, since many other amino acids form colored complexes resembling that of proline. The present experiments have shown that the isatin method can be made specific for proline by subsequent treatment of the chromatograms with dilute acid. The chief advantages of this new procedure are simplicity and degree of specificity achieved. Moreover, use of this method with synthetic medium M 150 precludes interference by approximately 60 substances of biological importance, including amino acids, purines, pyrimidines, vitamins, and accessory growth factors. In addition, this method does not require desalting, even though the concentration of inorganic ions in the synthetic medium is high. Binding of the proline chromogen to the paper and fading of the isatin colors of the other amino acids resulting from the acid treatment makes it possible to apply this method under conditions of poor separation and resolution on the chromatograms. Quantitative studies have established that the intensity of the color is linear over a concentration range of 1 to 16 μg of proline. This range is sufficient to measure the levels of proline normally encountered in natural substances. The application of this method for proline, in conjunction with the specific method for hydroxyproline previously reported(13), makes it possible to determine selectively each of these amino acids in biological materials.

Summary. A specific method for determining proline is reported, based on treatment of isatin-developed paper chromatograms with dilute acid. None of the 20 other amino acids of synthetic medium M 150, including hydroxyproline, reacted by this procedure. The proline chromogen exhibits an absorption maximum at 600 mμ. The color is remarkably stable and can be used for the quantitative determination of proline over the range of 1 to 16 μg.

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1. Pasieka, A. E., Morton, H. J., and Morgan, J. F., *J. Nat. Cancer Inst.*, 1956, v16, 995.

2. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.

3. Morgan, J. F., Campbell, M. E., and Morton, H. J., *J. Nat. Cancer Inst.*, 1955, v16, 557.

4. Hanks, J. H., and Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.

5. Acher, R., Fromageot, C., and Jutisz, M., *Biochim. et Biophys. Acta,* 1950, v5, 81.

6. Redfield, R. R., and Barron, E. S. G., *Arch. Biochem. and Biophys.,* 1952, v35, 443.

7. Smith, I., *Nature,* 1953, v171, 43.

8. Zacharius, R. M., Thompson, J. F., and Steward, F. C., *J. Am. Chem. Soc.,* 1952, v74, 2949.

9. McKee, H. S., and Urbach, G. E., *Austral. J. Biol. Sci.,* 1953, v6, 369.

10. Saifer, A., and Oreskes, I., *Science,* 1954, v119, 124.

11. Pasieka, A. E., and Morgan, J. F., *Biochim. et Biophys. Acta,* 1955, v18, 236.

12. ———, *ibid.,* 1956, v19, 366.

13. ———, PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 96.

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Reaction of Enzymes of *Lactobacillus bifidus* var. *pennsylvanicus* with Bifidus Factor: Effect of Monosaccharides. (22662)

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Activity as an essential growth factor for *L. bifidus* var. *pennsylvanicus*(1) is exhibited by a number of substances of high or low molecular weight, but all containing N-acetyl-D-glucosamine. Among the low molecular weight compounds with comparably high microbiological activity are such different types as the alkyl-N-acetyl-β-D-glucosaminides and 4-O-β-galactopyranosyl - N - acetyl - D-glucosamine; active large molecules include the mucopolysaccharides of human milk and the blood group substances. Free N-acetyl-D-glucosamine itself has some growth-promoting effect but is of the order of 1 to 2% as active as the bound forms. A cell-free extract from *L. bifidus* var. *pennsylvanicus*(2) inactivates the various forms of bifidus factor with release of N-acetyl-D-glucosamine and the other constituent monosaccharides. Watkins and Morgan(3) found that the action of an enzyme preparation from *Trichomonas foetus* on blood group substances was inhibited by the presence of certain simple saccharides acting, presumably, in competition with corresponding structures in the blood group substances. These experiments suggested that further information about the essential structure and mode of action of the "bifidus factor" might be obtained by studying the effect of simple sugars, particularly those which are components of the bifidus factor, on the activity of the enzyme preparation from *L. bifidus* var. *pennsylvanicus*. In other experiments the direct effect of these sugars on the growth of *L. bifidus* var. *pennsylvanicus* was tested. A number of sugars, notably D-galactose, N-acetyl-D-glucosamine and L-fucose were found to inhibit the enzymatic decomposition of the mucopolysaccharides from human milk and hog gastric mucin. Most striking was the very marked effect of fucose with the bifidus factor of human milk. An inhibitory effect of fucose could also be observed *in vivo*. It markedly inhibited the growth of *L. bifidus* var. *pennsylvanicus* when the source of the bifidus factor was human milk but not when other forms of bifidus factor were used.

Materials and methods. Most of the enzymatic studies and *in vivo* microbiological tests were made with the bifidus factor of human milk and hog gastric mucin. In addition to skimmed milk itself, a deproteinized non-dialyzable fraction and a slowly-dialyzable fraction were tested. Hog gastric mucin was suspended in water and centrifuged to remove any insoluble portion. No purified fractions were used. For comparison with these high molecular weight forms of bifidus factor N-acetyl-D-glucosamine, 4-O-β-galactopyranosyl-N-acetyl - D-glucosamine and ethyl - N-acetyl-β-D-glucosaminide were used. The ethyl glycoside was present as approximately 20% of an α-β mixture. The α-form is inert in the test. The preparation of the cell-free extract of *L. bifidus* var. *pennsylvanicus* has been described(2). The lyophilized material contains about 20% of protein. In most of the experiments the concentration of the enzyme preparation was 2 mg per ml for milk or preparations from milk and 5 mg per ml for mucin. These concentrations gave approximately equal rates of inactivation of the two substrates. Human milk was tested in a 1 to 4 dilution, mucin and the non-dialyzable preparations from milk at 5 mg per ml and the dialyzable milk fraction at 7.5 mg per ml. Inhibitors were tested first at a level of 4%. In later experiments active substances were studied at higher and lower concentrations. All experiments were carried out at pH 6.0 in phosphate buffer. Samples were incubated at 37° under toluene. Aliquots were withdrawn at desired intervals, diluted to a concentration suitable for assay and heated 10 minutes in boiling water to inactivate the en-

TABLE I. Effect of L-Fucose and D-Galactose on Microbiological Inactivation of and Release of N-Acetylhexosamine from Bifidus Factor by Means of Bifidus Enzyme.

		Control		Fucose		Galactose	
Substrate	Hr	Activity,* % loss	N-acetyl-hexosamine,† %	Activity, % loss	N-acetyl-hexosamine, %	Activity, % loss	N-acetyl-hexosamine, %
Non-dialyzable fraction from human milk	1	10	30	3	19	2	10
	6	72	68	20	44	27	42
	19	89	90	53	54	64	70
	48	93	93	60	75	78	90
Hog gastric mucin	1	28	24	10	10	4	8
	6	31	50	16	34	4	29
	19	48	72	31	51	25	34
	48	89	89	73	76	64	64

* Both substrates had an initial microbiological activity of approximately 2 units/mg.

† N-acetylhexosamine values are calculated as % of total amount which is released when substrate is completely inactivated.

zymes. N-acetylhexosamine was determined by a modification of the method of Aminoff *et al.*(4). The procedure for the microbiological assay of bifidus factor has been reported(5). The basal medium containing enzymatically digested casein corresponded to that of Hassinen *et al.*(6) modified by the addition of 1 g of "Tween 80" per liter of double strength medium. The effect of sugars, particularly fucose, was tested not only with *L. bifidus* var. *pennsylvanicus,* but also with the regular strain which does not require bifidus factor(7).

Results. Galactose and fucose inhibited the enzymatic splitting of the polysaccharides of both human milk and gastric mucin. A typical experiment is shown in Table I. Both release of N-acetylhexosamine and loss of microbiological activity were delayed. In other experiments the effect was shown to be proportional to the amount of galactose or fucose present. Similar results were obtained with lactose which is hydrolyzed by the bifidus enzyme preparation. The effect of N-acetyl-D-glucosamine could not be as conveniently studied. The amounts of sugar used as inhibitor were much larger than those which would be released from the substrates so that N-acetyl-hexosamine could not be measured. Also, since N-acetyl-D-glucosamine is a growth stimulant it was necessary to remove it from the reaction mixture by dialysis before estimation of microbiological activity. The results of the experiments carried out indicated that N-acetyl-D-glucosamine did act as an inhibitor of the bifidus enzyme.

Galactose and N-acetyl-D-glucosamine had a greater inhibiting action with mucin than with human milk fractions. In many experiments in which the release of N-acetylhexosamine was followed the galactose effect with human milk was limited chiefly to the first hours of the experiment whereas with mucin it was quantitatively greater and more prolonged. With N-acetyl-D-glucosamine the rate of inactivation of milk polysaccharides was only moderately slower than that of the controls. With mucin the effect of N-acetyl-D-glucosamine seemed more persistent. Even after prolonged incubation about 30% of the initial activity was left while the control was completely inactive (Table II). Fucose, however, was most active with the milk polysaccharides and the extent of inhibition was greater than any observed effect of galactose or N-acetyl-D-glucosamine. This observation was confirmed in repeated tests. The effect appeared to depend primarily on the concentration of fucose rather than on a competitive action with the substrate. Marked inhibition of release of N-acetylhexosamine had been shown with 4% fucose and 0.5% of the non-dialyzable fraction of milk. With lower levels of fucose, 0.06 to 2%, inhibition was demonstrated with levels of substrate from 0.15 to 5%.

Of a number of other sugars tested only methyl-α-L-fucopyranoside and 1-acetamino-

TABLE II. Effect of N-acetyl-D-glucosamine on Inactivation of Bifidus Factor by Bifidus Enzyme.

Substrate	Hr	Activity (%)	
		Control	N-ac-glucosamine
Non-dialyzable fraction from human milk	1	79	88
	6	32	41
	24	17	30
	48	<5	11
	80	<5	<5
Hog gastric mucin	1	82	89
	6	62	62
	24	49	57
	48	<5	56
	80	<5	31

lactose were active. D-Glucose, L-fructose, L-rhamnose and L-arabinose were essentially without effect.

In the microbiological assay of samples from the enzyme tests it became apparent that, in some cases, the presence of L-fucose was inhibiting the growth of *L. bifidus* var. *pennsylvanicus*. When this observation was tested systematically, with varying amounts of fucose in the medium and with different forms of bifidus factor, it was found that inhibition occurred when bifidus factor was supplied as human milk or fractions from human milk, but not when other forms of bifidus factor were used (Table III). The effect was appreciable with 0.1% of fucose in the medium and increased with larger amounts of fucose. The effect was greater with suboptimal levels of bifidus factor. Fucose was equally effective whether it was autoclaved with the medium or added aseptically to previously autoclaved medium. Methyl-α-L-fucopyranoside which inhibited the bifidus enzyme had no effect *in vivo*. Up to a level of 0.5% fucose did not inhibit or retard the growth of the regular, non-milk-requiring strain of *L. bifidus*. The medium for *L. bifidus* contains lactose as carbohydrate. The organism does not grow as well with either glucose or galactose. These monosaccharides were tested for possible inhibitory effect. No inhibition was found when they were added in concentration up to 1%.

Discussion. The mucopolysaccharide of human milk, like the blood group substances, contains N-acetyl-D-hexosamine, D-galactose and L-fucose. It differs from the blood group substances in that it contains glucose and that the hexosamine is almost entirely glucosamine while all of the blood group substances have an appreciable proportion of galactosamine. Structure of a hexasaccharide unit of the milk polysaccharide has been reported by Kuhn(8) but the position of this unit in the substance as it occurs in milk is still to be worked out. The hexasaccharide contains 2 molecules of L-fucose in α-fucopyranosidic linkage. The L-fucose of the blood group substances is also present as an α-fucopyranoside, but its position in the whole oligosaccharide is not known and is probably not the same for all of the blood group substances (*cf* 9). Kuhn and Osman(10) have shown that the fucose-containing oligosaccharides of human milk have very low blood group O(H) activity not only in comparison with the blood group substance but also in comparison with simple α-fucopyranosides and L-fucopyranose.

In earlier experiments with the *L. bifidus* enzyme preparation loss of microbiological activity was known to be accompanied by increase of reducing sugar, and release of N-acetyl-D-glucosamine was revealed chromatographically. In the present experiments release of N-acetyl-D-hexosamine in a form measurable by the Morgan-Elson reagent, was followed quantitatively and was found to parallel closely the degree of microbiological inactivation with enzyme alone or in the presence of galactose or fucose. Approximately 75 γ of N-acetyl-D-glucosamine was released for each unit of activity lost. Of the 4 components of the mucopolysaccharides tested only glucose was without effect. The other sugars exhibited the type of inhibition which might be expected from the presence of an

TABLE III. Growth of *L. bifidus* var. *pennsylvanicus* with and without Fucose.

Supplement of bifidus factor	Acid production (ml of N/10)	
	No fucose	0.15% fucose
Skimmed human milk, .06 ml	8.5	5.3
Non-dialyzable fraction from human milk, .5 mg	10.5	8.7
Dialyzable fraction from human milk, 1.5 mg	7.5	5.0
Hog gastric mucin, .5 mg	9.7	9.4

end product. Only in the case of fucose with the milk polysaccharide was there indication of a more specific effect. *In vivo* only fucose exerted an inhibitory effect. This inhibition was limited to the milk polysaccharide and could not be demonstrated with hog gastric mucin or low molecular weight forms of bifidus factor. Neither did fucose have any effect on the growth of strains of *L. bifidus* which do not require the bifidus factor. The position of fucose in the milk polysaccharide appears to give it distinctive properties with respect to activity as bifidus factor.

Summary. 1. The effect of the constituent monosaccharides on the enzymatic inactivation of high molecular forms of bifidus factor has been studied. The most marked effect was observed with fucose when it was used with human milk or purified fractions from human milk. 2. The significance of this effect of fucose on the inactivation of the bifidus factor *in vitro* was emphasized by the experiments *in vivo*. L-Fucose markedly inhibited growth of *L. bifidus* var. *pennsylvanicus* when the source of bifidus factor was human milk, but not when it was supplied as mucin or the simple N-acetyl-D-glucosamine-containing compounds.

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1. György, P., *Pediatrics*, 1953, v11, 98.
2. György, P., Rose, C. S., and Springer, G., *J. Lab. Clin. Med.*, 1954, v43, 543.
3. Watkins, W. M., and Morgan, W. T. J., *Nature*, 1955, v175, 676.
4. Aminoff, D., Morgan, W. T. J., and Watkins, W. M., *Biochem. J.*, 1952, v51, 379.
5. György, P., Norris, R. F., and Rose, C. S., *Arch. Biochem. and Biophys.*, 1954, v48, 193.
6. Hassinen, J. B., Durbin, G. T., Tomarelli, R. M., and Bernhart, F. W., *J. Bacteriol.*, 1951, v62, 771.
7. Norris, R. F., Flanders, T., Tomarelli, R. M., and György, P., *ibid.*, 1951, v60, 681.
8. Kuhn, R., *Angew. Chemie*, 1955, v67, 184.
9. Kabat, E., *Blood Group Substances; Their Chemistry and Immunochemistry*, 1955 New York.
10. Kuhn, R. and Osman, H. G., *Z. Physiol. Chemie*, 1956, v303, 1.

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Chemical Inhibitors of Theiler's Virus.* (22663)

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Certain amino acids, nucleoprotein derivatives and analogues as well as various other compounds, previously have been reported to inhibit propagation of Theiler's GD VII virus, *in vitro*(1-5). In the present study some additional chemicals have been tested. A preliminary report was given elsewhere(6).

Methods. Tissue cultures of minced brain tissue from newborn mice were made in 50 ml stoppered, Erlenmeyer flasks. Each flask contained 50-100 mg of tissue in 3 ml Simms' solution at pH 8-9 and approximately 100 intracerebral MLD_{50} of a tissue culture passage strain of Theiler's GD VII mouse encephalomyelitis virus. After incubation at 35-36°C for 2 days the pooled supernatant fluids obtained by centrifugation of the contents of 3 flasks were tested for viral content by hemagglutination of human red cells. Ordinarily titers of 1280-2560 are obtained by this procedure. The structure of the hydroxycytidine used as prepared in this laboratory has not been established with certainty. The Metuchen was kindly furnished by Dr. F. M. Berger, Wallace Laboratories, the thiosemicarbazone compounds by Dr. R. L. Thompson, Sterling-Winthrop Research Institute.

Results. Table I gives results of tests with various chemicals. Of the substituted pyrimidine nucleosides, the cytidine compounds are slightly more inhibitory than are the corresponding derivatives of uridine. Some of the naturally occurring deoxyribosyl nucleo-

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TABLE I. Percentage Reduction of Hemagglutination Titers of Virus in Presence of Certain Compounds.

Compound		% reduction of titer	
Nucleosides		.5 mg/ml	.1 mg/ml
5-bromocytidine		98	0
5-chlorocytidine		95	0
5-hydroxycytidine		90	50
5-hydroxyuridine		75	0
deoxyadenosine		75	0
deoxycytidine		0	—
deoxyguanosine		75	50
		3 mg/ml	1 mg/ml
5-bromodeoxyuridine		—	0
5-hydroxydeoxyuridine		—	0
thymidine		0-50	0
deoxyadenosine		100	95
deoxycytidine		50	0
deoxyuridine		—	0
Pyrimidine derivatives			
deoxycytidine-5′-phosphate		—	0
2-amino-4 methylpyrimidine		100	0
4,6-diamino-2-thiopyrimidine		90	85
2,4-dichloropyrimidine		0	—
2,4-dichloro-6-methylpyrimidine		0	—
5-bromouracil		—	50
2-thiouracil		50	0
propylthiouracil		100	75
5-methyl-2-thiouracil		90	0
6-methyl-2-thiouracil		100	0
Thiosemicarbazones		.1 mg/ml	.01 mg/ml
butane-2,3-dione-2-methoxime 3-		85	50
2-thenaldehyde-		50	0
5-bromo-2-thenaldehyde-		—	0
2,3-butane oxime-		—	0
Other chemicals	.1 mg/ml	.05 mg/ml	.005 mg/ml
chlorpromazine	—	100	50
quinine dihydrochloride	100	—	0
bacitracin	90	50	—
polymyxin	50	—	—
penicillin			
erythromycin			
mycostatin	0-50	—	—

sides were inhibitory as were also some of the ribosyl nucleosides(3). The compounds listed under pyrimidine derivatives varied in their capacity to inhibit virus. Two of the 4 thiosemicarbazones tested were inhibitory. The more active of the 2 was previously found by others to be the most inhibitory for vaccinia virus(7). Of the other chemicals used, chlorpromazine and one not listed in Table I, Metuchen (2,3 - diethyl - 3 - propanediol dicarbamate), are known to have effects on the CNS. The latter compound was not active at a concentration of 0.1 mg/ml but did give 75% reduction in hemagglutination titer at a concentration of 1 mg/ml and complete inhibition at 3 mg/ml. Chlorpromazine was as inhibitory as any drug ever tested in our system. An *in vivo* trial of the drug was made in the following manner. Groups of 10 albino Swiss mice (10-15 g) were each given intraperitoneally (i.p.) 1 mg of chlorpromazine daily (2 mg daily is lethal). A control group of mice received saline instead of chlorpromazine. Seven hours after the first dose of drug and immediately prior to the second dose, each mouse was given an intracerebral (i.c.) injection of 0.03 ml of tissue culture virus (approx. 1-10 MLD_{50}). The test mice died 3 to 8 days after virus injection as follows 3,3,3,3,7,7,7,7,7,8; deaths of control mice were at 4,7,7,7,8,9,10 days and 2 survived. In a second experiment drug treatment was given as above daily beginning on the third day after virus inoculation. Mice died on the days indicated after virus injections; test mice, 5,5,6,7,7,10,11, 3 survivors; control mice, 4,6,7,7,10,10,10, 2 survivors. The drug did not protect mice from viral death. The early deaths in the first experiment possibly resulted from a combination of viral effect and drug toxicity.

Bacitracin polymyxin, penicillin and erythromycin were found to inhibit virus; streptomycin, tetracycline, oxytetracycline and chloramphenicol did not inhibit. Mycostatin irregularly gave inhibition; possibly this irregularity is associated with the poor solubility of the drug. Polymyxin was used to treat mice which had received 1 MLD_{50} of virus i.c. 3 days previously. The mice were given 0.2 mg i.p. daily; control mice received saline. Control and test mice died 5-9 days after virus injection. The drug did not affect the infection in mice. Under the conditions of this experiment 0.3 mg daily of polymyxin was a fatal dose.

Inhibition of virus by 5-hydroxyuridine was reversed by uridine-5′-phosphate as shown in Table II. Comparable reversal of this inhibition also occurred with uridine(3).

Glutamine has been found by others(8) to be necessary for the propagation of poliovirus in HeLa cell tissue cultures. This compound inhibited GD VII virus in our tissue culture system. The hemagglutination titer was reduced 75% by a concentration of 0.1 mg/ml.

Discussion. Uridine does not inhibit virus and partially reverses inhibition produced by some substituted uridine nucleosides(3). As shown above uridine-5′-phosphate also reverses the inhibitory effect of the nucleoside derivative, 5-hydroxyuridine. The greater activity of cytidine derivatives in comparison with uridine derivatives as shown here is similar to the findings for *Neurospora*(9). Although chlorpromazine was active, *in vitro*, it failed to influence the infection in mice. The quantity used in the tests was nearly the maximal amount tolerated since it was found in preliminary studies that 2 mg daily was a lethal dose. The dose of 1 mg used resulted in generalized muscular weakness from which recovery was gradual and not complete as long as 24 hours later. The drugs tested probably did not act by interfering directly with the hemagglutination test since control tests with representative drugs from each group and virus incubated for 1 hour at room temperature gave the same hemagglutination titer as virus alone with one partial exception; the RBC sediment pattern in the presence of 0.1 mg/ml quinine dihydrochloride did not have as definite an endpoint as the control tube with virus.

TABLE II. Reversal of 5-Hydroxyuridine Inhibition by Uridine-5′-phosphate.

	% reduction of hemagglutination titer
5-hydroxyuridine, .5 mg/ml	75
Uridine-5′-phosphate, .5 mg/ml	0
5-hydroxyuridine, .5 mg/ml + uridine-5′-phosphate, .5 mg/ml	50
5-hydroxyuridine, .5 mg/ml + uridine-5′-phosphate, 1 mg/ml	0

Summary. Certain pyrimidine-related-compounds inhibit Theiler's virus, *in vitro*. Inhibition by 5-hydroxyuridine is partially reversed by uridine-5′-phosphate. Of various other chemicals found to inhibit virus, *in vitro*, chlorpromazine and polymyxin did not protect mice from viral infection.

1. Pearson, H. E., *J. Immunol.*, 1950, v64, 447.

2. Pearson, H. E., Lagerborg, D. L., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 409.

3. Visser, D. W., Lagerborg, D. L., and Pearson, H. E., *ibid.*, 1952, v79, 571.

4. Pearson, H. E., and Lagerborg, D. L., *J. Bact.*, 1955, v69, 193.

5. Pearson, H. E., Lagerborg, D. L., Winzler, R. J., and Visser, D. W., *ibid.*, 1955, v69, 225.

6. Pearson, H. E., Lagerborg, D. L., and Visser, D. W., *Fed. Proc.*, 1956, v15, 607.

7. Thompson, R. L., Davis, J., Russell, P. B., and Hitchings, G. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 496.

8. Eagle, H., and Habel, K., *Fed. Proc.*, 1956, v15, 585.

9. Visser, D. W., *Antimetabolites and Cancer, AAAS Sympos.* 1955, 47.

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Relation of Endocrines and Clearing Factor Inhibitors to Hyperlipemia in Fasted Animals.*† (22664)

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A variety of substances inhibit the clearing action of lipoprotein lipase *in vitro*. These include protamine(1), polylysine(2), diisopropylfluorophosphate (DFP)(3), iodinated thyronines(4), Na cholate(5), and an inhibitor (LM) present in plasma of rats receiving cortisone(6). Of these protamine(7), polylysine (2), Na cholate(5), and LM(8) have been reported to produce hyperlipemia when injected

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into intact animals, presumably by inactivating lipoprotein lipase *in vivo*. LM, apparently produced by the pituitary, induced hyperlipemia in adrenalectomized and hypophysectomized rats and may be considered to either activate the fat depots or to inhibit lipoprotein lipase. It was of interest therefore to establish whether other hyperlipemia inducing agents act similarly. The failure of these to produce hyperlipemia in the absence of the adrenals or pituitary would suggest that the effect in intact animals could not be due to direct inactivation of lipoprotein lipase *in vivo* but rather the result of mediation through release of LM.

Materials and methods. To demonstrate the effect of food intake on the hyperlipemia inducing action of LM, 6 groups of 5 male and 5 female Wistar strain rats weighing 125-150 g were used. Three groups were maintained on the laboratory basal diet of Purina Chow checkers containing 5% fat up to the time of injection (Category A) and 3 groups were fasted for 20 hours preceding injection (Category B). Neither category received food from the time of injection until bled. One group of each received 5 mg LM/kg either IV, IP or IM. Blood samples were obtained at the end of 2 hours. For the other rat studies all animals were fasted 18-24 hours and all injections were made into the recurrent saphenous vein. Solutions of compounds to be tested were freshly prepared in physiological salt solution just prior to use and the concentration was adjusted so that 0.1 ml/100 g of body weight was administered. Blood was obtained by cardiac puncture through the open chest while the animal was under light ether anesthesia. At this time it was verified that the stomach of fasted rats contained no food. The blood was mixed with 0.1 M Na citrate (1:9) and centrifuged in a constant temperature room (5° ± 1°C) until separation of the plasma occurred. Total cholesterol was determined by the method of Schoenheimer-Sperry(9), total fatty acids by that of Stoddard and Drury(10), and lipid P by that of Fiske and SubbaRow(11). Adrenalectomized rats maintained on Purina Chow checkers and 0.9% NaCl in their drinking water, and hypophysectomized rats fed white bread, Pard dog food, and oranges were used 10-14 days after operation. Completeness of adrenalectomy and hypophysectomy was checked in each rat at autopsy. In another study 3 male and 3 female mongrel dogs previously fasted for 18 hours were injected intravenously as described below. The previous diet of these dogs contained approximately 5% fat. Lipid values were determined, as described for the rats, on blood samples obtained from the recurrent saphenous vein before, during, and after each experiment. At least 7 days were allowed between studies and blood samples were drawn during these intervals.

Protamine sulfate (Lilly) of fish origin was administered to 20 fasted intact rats, 20 fasted adrenalectomized rats, 20 fasted hypophysectomized rats and to the dogs in doses of 10 mg/kg. Blood samples were obtained from rats 1 hour after the injection and from dogs at hourly intervals for 6 hours. DFP supplied by Drs. John W. Gofman and Bernard Shore was administered to dogs in doses of 0.05, 0.10, and 1 mg kg and to 7 fasted intact rats, 7 fasted adrenalectomized rats and 7 fasted hypophysectomized rats in a dose of 1 mg/kg. Blood samples were obtained from the dogs at hourly intervals for the first 6 hours after injection and at the 24th hour and from rats 2 hours after injection. Pseudomonas polysaccharide (Piromen, Travenol Laboratories) was administered to 20 fasted intact rats, 20 fasted adrenalectomized rats and 20 fasted hypophysectomized rats in a dose of 50 μg/kg. Blood samples were taken 2 hours after the injection. The lipid mobilizer (LM) was prepared from the plasma of cortisonized horses as previously described(8) and was administered to 20 fasted intact rats, 20 fasted adrenalectomized rats and 20 fasted hypophysectomized rats and to the dogs in 1 mg kg doses. Blood samples from rats receiving LM were obtained 1 hour after injection and from dogs at hourly intervals for the first 6 hours and at the 24th hour.

Results. Table I shows that injections of LM produced hypercholesterolemia only

TABLE I. Effect of Food Intake on Hypercholesterolemia Produced by LM in Rats.

	Total plasma cholesterol (mg %)* IV	IM	IP
Fasted	184.3 ± 3.5	148.6 ± 3.2	168.6 ± 3.6
Non-fasted	60.0 ± 2.6	61.8 ± 2.0	64.0 ± 1.8

* Avg 10 rats/group ± S.D.

when injected into fasted rats but not in those which had received food up to the time of injection. The hyperlipemia in fasted rats receiving LM is illustrated in Fig. 1 and the values for each lipid component represent the averages for 20 rats (S.D. ± 5%). The hyperlipemic action did not depend upon the presence of either adrenals or pituitary. Fig. 2 shows the degree of hyperlipemia in rats receiving protamine. Similar results were obtained with rats administered either DFP or piromen. As with LM, in intact rats they produced a 2-3 fold elevation of all classes of lipids and a marked increase in optical density. In contrast, however, neither protamine, DFP nor piromen produced hyperlipemia in the absence of either adrenals or pituitary. The rats administered the DFP had convulsions and those given piromen had elevated rectal temperatures of at least 2.5-3°C. In the dogs receiving LM there was a 2-3 fold increase in all lipid components in 90-180 minutes which persisted for at least 3 hours. The plasma lipid values were in the normal range 24 hours after injection. Protamine administered to these same dogs at a later date also produced an increase in plasma lipid values, but the effect was less intense, of shorter duration, and the dose required was 10 times greater than that of LM. Similar administration of DFP to these dogs also produced hyperlipemia resembling that produced by protamine, but this occurred only after convulsant doses. The hyperlipemia did not appear after the dogs were administered non-convulsant doses of DFP although they exhibited unequivocal signs of anticholinesterase effects.

Discussion. LM induced hyperlipemia only in animals that were primed for lipid mobilization by starvation. It should be pointed out that all animals used in our studies were maintained on a low fat diet. In previous investigations of lipid mobilization diet, degree of fasting, or pretreatment with glucose had marked influences on the response (12). Of the many substances that produce hyperlipemia, particular significance has been attached to protamine(7) because this compound is considered to be a specific antagonist of heparin. Therefore the hyperlipemia it induces has been attributed to inactivation of lipoprotein lipase and has been presented as

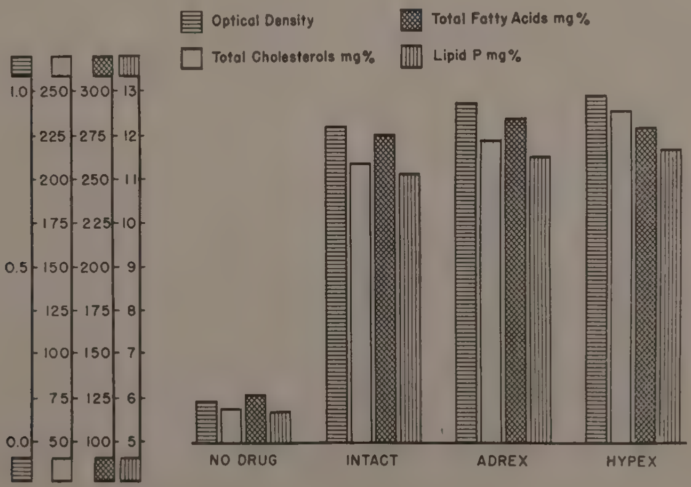

FIG. 1. Hyperlipemia by 1 mg LM/kg IV in rats (1 hr $\overline{p}$ inj.).

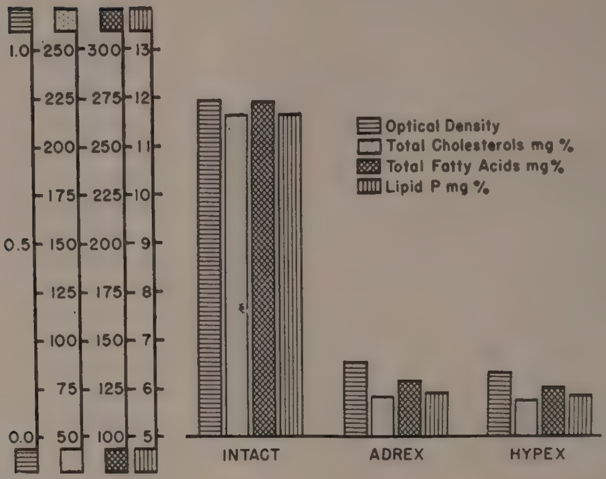

FIG. 2. Hyperlipemia by 10 mg protamine SO_4/kg IV in rats (1 hr $\overline{p}$ inj.).

evidence for the presence of heparin in the enzyme. However, the effective dose of protamine(7) is far in excess of that needed to neutralize the amounts of circulating heparin that can be detected by present methods and is within the toxic range that activates the pituitary and adrenals(13). Of particular significance is the large dose of protamine required to produce the hyperlipemia which was less intense than that produced by LM. Administration of larger doses resulted in more intense hyperlipemia which was accompanied by gross hematuria, prolonged shock with terminal asphyxial convulsions. The failure of protamine to produce hyperlipemia in adrenalectomized and hypophysectomized rats establishes that the action was not due to a direct effect on lipoprotein lipase but was mediated through the adrenal-pituitary system. Spitzer has also presented indirect evidence that protamine hyperlipemia involves more than anti-heparin activity(14). Shore has demonstrated that DFP is a potent inhibitor of lipoprotein lipase *in vitro*(3). Therefore administration of DFP in amounts to achieve similar concentrations in the plasma should result in hyperlipemia as a consequence of inactivation *in vivo*. We could observe no hyperlipemia in intact animals until convulsions had occurred which required administration of several times this amount. Furthermore, the hyperlipemia did not occur in adrenalectomized or hypophysectomized rats receiving the same doses and exhibiting convulsions. These results establish that the hyperlipemia due to DFP is a non-specific one mediated through the adrenals and pituitary and is not related to its anti-lipoprotein lipase activity. Since LM was obtained only from animals with intact pituitaries and induced hyperlipemia in the absence of either the adrenals or pituitary, it may be assumed to be released through the pituitary and to be the mediator through which stressors act as hyperlipemic agents. Administration of heparin releases lipoprotein lipase, but more direct evidence than hyperlipemia produced by protamine or DFP is needed to establish the presence of a heparin moiety in this enzyme or that it is involved in the physiologic or pathologic transport of lipids. The results with piromen establish that this agent also induced hyperlipemia by activating the pituitary and adrenals. Piromen has not been shown to inactivate lipoprotein lipase and therefore furnishes an excellent example of a non-specific stressor activating the pituitary-adrenal system to produce hyperlipemia.

Summary. 1. LM produced hyperlipemia only in animals primed for lipid mobilization. 2. Intravenous injection of toxic doses of protamine sulfate, convulsant doses of diisopropylfluorophosphate and pyrogenic doses of piromen produced hyperlipemia in intact animals. 3. The same doses of these substances injected into adrenalectomized and hypophysectomized rats did not produce hyperlipemia. 4. The hyperlipemic action of protamine or DFP is not attributable to direct inhibition of lipoprotein lipase but to a secondary effect mediated through the adrenals and pituitary. 5. The hyperlipemic action of protamine, DFP, pyrogens and possibly other stressors is best explained by release of LM.

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1. Brown, R. K., Boyle, E., and Anfinsen, C. B., *J. Biol. Chem.*, 1953, v204, 423.

2. Sten, Y., and DeVries, A., *Arch. Int. Pharmacodyn.*, 1955, vCII, No. 3, 274.

3. Shore, B., Nichols, H. V., and Freeman, N. K., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 216.

4. Shore, B., *ibid.*, 1955, v90, 415.

5. Friedman, M., and Byers, S. O., *ibid.*, 1951, v78, 528.

6. Seifter, J., and Baeder, D. H., *ibid.*, 1954, v86, 709.

7. Bragdon, J. H., and Havel, R. J., *Am. J. Physiol.*, 1954, v177, 128.

8. Seifter, J., and Baeder, D. H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 42.

9. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, 1934, v106, 745.

10. Stoddard, J. L., and Drury, P. E., *ibid.*, 1929, v84, 741.

11. Fiske, C. H., and SubbaRow, Y., *ibid.*, 1925, v66, 375.

12. Levin, L., and Farber, R. K., *Recent Prog. in Hormone Research*, 1952, vVII, 399.

13. Vartiainen, I., and Maible, A., *J. Lab. and Clin. Med.*, 1941, v26, 1416.

14. Spitzer, J., *Am. J. Physiol.*, 1953, v174, 43.

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Evaluation of Germicidal Efficiencies of a Group of Antibiotics Tested by Tissue Culture Technic. (22665)

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An improved one-step tissue toxicity technic was proposed recently(1) for evaluation of germicides intended for clinical application.

The germicides were tested for their effect on living embryonic chick heart tissue fragments as well as for their ability to kill bacteria. A number known as the Toxicity Index was calculated from the results, which was defined as the ratio of highest dilution of germicide required to prevent growth of the tissue fragments in 10 minutes to highest dilution required to kill the test bacteria in the same period and under similar conditions. Theoretically an index less than one means that the germicide is more toxic to the test organism than to the embryonic tissue fragments; an index greater than one means that the germicide is more toxic to the tissue fragments than to the bacteria. The smaller the index the more nearly perfect the chemotherapeutic agent. There is no relation whatsoever between a phenol coefficient and a toxicity index.

The same procedure was followed for evaluation of the germicidal efficiencies of a group of antibiotics including: Bacitracin, Chloramphenicol (Chloromycetin) Penicillin G sodium, Polymyxin B sulphate, Streptomycin sulfate, and Terramycin HCl.

Aureomycin (Chlortetracycline HCl) and Achromycin (Tetracycline HCl) precipitated from saline shortly after being prepared and could not be evaluated. Likewise, Erythromycin failed to dissolve in saline.

Methods and materials. Embryonic chick heart tissue fragments from embryos 12 days old were used. Hearts were selected because of the ease by which a constant source of tissue could be obtained. The hearts were removed from the embryos and minced into fragments 0.5 to 1.0 mm in diameter by means of a sharp knife. The fragments were washed twice in normal saline, then resuspended in saline in the proportion of one heart per ml. This suspension of tissue fragments was handled like a broth culture of bacteria.

Micrococcus pyogenes var. *aureus* is generally used as the organism in germicide testing but since it liquefied plasma it could not be used here; *M. pyogenes* var. *albus* was used instead. Unless the fragments are embedded in coagulated plasma, microscopic observations of Carrel flasks for tissue proliferation could not be made.

The culture was grown on FDA nutrient agar slants incubated at 37°C and transferred daily. The organisms were washed from a 24-hour culture with saline and the suspension standardized in a photometer to correspond to the same turbidity as a No. 1 McFarland nephelometer standard.

For each test, a series of 8 dilutions of antibiotic was prepared in screw cap test tubes measuring 20 x 150 mm. The dilutions were prepared in normal saline according to the following scale of dilutions: Dilutions from 10 to 100 at intervals of 10; from 100 to 200 at intervals of 20; from 200 to 500 at intervals of 50; from 500 to 1000 at intervals of 100; from 1000 to 50,000 at intervals of 1000.

In another series, each screw cap test tube contained a mixture of 1.5 ml saline, 0.5 ml bacterial suspension, and 0.5 ml tissue suspension, making a total volume of 2.5 ml in each tube.

All tubes were placed in a 37°C water bath containing a shaking device and kept in constant agitation for 30 minutes. At the end of 30 minutes, 2.5 ml of the first antibiotic dilution was pipetted into one of the tubes containing the mixture of saline, bacteria, and tissue fragments, making a final volume of 5 ml. The cap was screwed firmly in place and the tube shaken vigorously to wet completely the inner surface before returning it to the water bath. This same procedure was continued at 1-minute intervals for the remaining dilutions in the series. The tubes were kept in agitation in the water bath throughout the test period.

At the end of 9½ minutes, the first tube of the series was removed from the bath and the supernatant aspirated from the fragments. At the end of exactly 10 minutes, the tube was filled with about 25 ml saline to dilute the small amount of antibiotic still remaining in the tube, then set aside. The remaining tubes were treated at 1-minute intervals in the same manner. The fragments were washed 2 more times, then embedded in plasma in Carrel flasks.

The embedding medium consisted of 0.3 ml heparinized rabbit plasma, 0.5 ml Tyrode's solution, and 0.4 ml embryonic extract.* After coagulation of the plasma medium, 1 ml embryonic extract was added as nutrient. Flasks were plugged with paraffined corks and incubated at 37°C for 120 hours before making final readings.

Each flask contained a total of approximately 35 pieces of tissue measuring 0.5 to 1.0 mm in diameter. After all ingredients were added, the Carrel flasks were gently rotated to insure uniform distribution of the tissue fragments before coagulation of the plasma occurred.

Results. The highest dilution of antibiotic that showed no growth of bacteria in the Carrel flask was taken as the killing dilution. The highest dilution of antibiotic that showed no growth of tissue in the Carrel flask was taken as the killing dilution. Several series generally were necessary to determine the killing dilution for both bacteria and tissue. The toxicity index was calculated by dividing the highest dilution of antibiotic required to kill the tissue fragments by the highest dilution required to kill the test organism. Results of tests on a number of well-known germicides (1) have shown iodine to exhibit the greatest degree of germicidal efficiency with a toxicity index of 0.10. This figure indicates that under the conditions of the test iodine is 10 times more toxic to the bacteria than to the chick heart tissue fragments, a remarkable figure for a germicide. Since iodine exhibited the greatest degree of efficiency of the germicides tested, it was used as the basis of com-

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* The extract was prepared by mixing 10-day-old minced chick embryos with 5 times its volume of Tyrode's solution and centrifugated at 3000 rpm for 30 minutes. The clear supernatant is the embryonic extract.

TABLE I. Highest Killing Dilution of Antibiotic for Embryonic Chick Heart Tissue and *Micrococcus pyogenes* var. *albus* and Corresponding Toxicity Index.

Antibiotic	Tissue (A)	Bacteria (B)	Toxicity index (A/B)
Iodine	1:2000	1:20,000	.10
Penicillin G sodium	1:12	1:5000	.0024
Streptomycin sulfate	1:20 growth	1:1000	<.02
Polymyxin B sulfate	1:50 growth	1:80	<.63
Terramycin	1:600	1:900	.67
Bacitracin	1:10 growth	1:10 growth	—
Chloramphenicol	1:100 growth	1:100 growth	—

parison for a number of well-known antibiotics. Of the antibiotics tested (Table I) penicillin G sodium exhibited the greatest degree of germicidal efficiency, combining high potency with low tissue toxicity. A toxicity index of 0.0024 indicated that penicillin was more than 400 times more toxic to the test bacteria than to the tissue cells, and approximately 42 times more efficient than iodine. Terramycin with an index of 0.67 was inferior to iodine as a germicide. Streptomycin sulfate was nontoxic to tissue in the highest concentration used and gave an index less than 0.02. A more concentrated solution could not be prepared without sacrificing accuracy. Likewise, Polymyxin B sulfate was nontoxic to tissue in the highest concentration used but killed the test organism in a dilution of 1:80 giving an index of less than 0.63. Chloromycetin and Bacitracin had no effect on tissue or bacteria in the concentrations used which would indicate that their actions were bacteriostatic rather than bactericidal.

Summary. A new method was proposed recently for the evaluation of germicides intended for clinical application. Iodine, with a toxicity index of 0.10 exhibited the greatest degree of germicidal efficiency. The same procedure was followed for the evaluation of a group of antibiotics. Penicillin G sodium gave an index of 0.0024 which indicated that it was more than 400 times more toxic to the test organism, *Micrococcus pyogenes* var. *albus*, than to the tissue cells, and approximately 42 times more efficient than iodine. Results on other antibiotics in order of decreasing efficiency are: Streptomycin sulfate, 0.02—; Polymyxin B sulfate, 0.63—, Terramycin, 0.67. Bacitracin and Chloramphenicol were bacteriostatic and could not be evaluated. Likewise, Aureomycin HCl, Achromycin HCl, and Erythromycin could not be evaluated; the former two precipitated from solution shortly after being prepared, and the latter failed to dissolve in saline.

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1. Salle, A. J., *Applied Microbiol.*, 1955, v3, 63.

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Quantitative Determination of C-Reactive Protein by Complement Fixation.[*][†][‡] (22666)

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Interest has been growing in the measurement of serum levels of C-reactive protein as an indicator of inflammatory processes, in particular, those associated with rheumatic fever, myocardial infarction, and cancer. The original determination of this substance(1) was based on the precipitate it forms with pneumococcal C-polysaccharide. Lack of sensitivity led to an immunochemical method, the reaction between C-reactive protein antigen and specific rabbit antibody. The degree of reaction is visually estimated from the quantity of specific precipitate(2,3). More recently, estimation by complement fixation (4) and gel diffusion(5) have been reported. In this communication we describe our modifications of the complement fixation method of Muschel and Weatherwax and the theoretical and practical reasons for them. Our purpose was 2-fold: to establish an objective index of the serum level of C-reactive protein, and to control the variables inherent in the quantitative determination of antigens by complement fixation.

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[*] Presented before Am. Assn. Immunol., Atlantic City, N. J., April 17, 1956.

[†] We wish to express our appreciation to Dr. Alfred Halpern and Schieffelin and Co. for their cooperation, to Dr. Maclyn McCarty for a specimen of pneumococcal C-polysaccharide, and to Mr. Nicholas Alonzo, Mr. Bernard Schiffer, and Mrs. Evelyn Abeshouse for their excellent technical assistance.

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The method described was designed to measure C-reactive protein in the range determined in the microprecipitation method, using approximately the same quantity of antiserum, and furnishing the result within a short time.

Materials and methods. Human test sera. Test sera were inactivated at 56°C for 10 minutes(4). Specimens so treated showed no residual complement activity at a dilution of 1:10. No loss was observed in the standard CRP serum after this treatment.

Rabbit anti-CRP serum. Native antiserum preserved with sodium azide (1:1000) was obtained from Schieffelin and Co. Antibodies to normal serum components were absorbed by adding lyophilized normal human serum, 0.05 g/ml. After incubation at 40°C for 2 hours and 4°C for 2 days, a small quantity of precipitate formed. The treatment to this point was based on the procedure Schieffelin and Co. employs to make its reagent specific. Removal of the precipitate by the usual centrifugation results in an antiserum which is anticomplementary because of the presence of soluble immune complexes. This is the source of the anticomplementary property reported by Muschel and Weatherwax(4). The absorbed antiserum was centrifuged in the #40 rotor of a Model L Spinco ultracentrifuge for 2 hours at 35,000 rpm (110,000 x g). The floating lipid layer (4 mm) was rejected and the next 42 mm of fluid was withdrawn in several portions which were subsequently combined. The lowermost 12 mm of clear supernatant was then removed and kept separately. Examination of anticomplementary property showed that this increased with sample depth, but the recombined specimen described was relatively free of this interference (hemolysis greater than 55% with two 50% hemolytic units of complement at antiserum dilution 1:10). The antiserum, preserved with merthiolate (1:10,000) and stored at —20°C, is used without inactivation, since its complement is removed during the absorption. Antiserum from the lowest fifth of the tube was too anticomplementary for complement fixation tests but was useful for microprecipitation and gel diffusion analyses.

Standard CRP serum. A specimen of serum with a high CRP level was stored in small quantities at —20°C and left at 4°C after being thawed. This standard was assigned a titer of 160 after comparison with an earlier standard which had been arbitrarily set at 100. The quantity of CRP in the standard, determined by precipitation with C-polysaccharide according to the method of Wood and McCarty(3) was 280 μg/ml.

Complement fixation experiments. Reagents and procedure have been described in detail(6).

Evaluation of reactions. The results are expressed as the quantity of CRP serum (antigen) required to obtain 50% hemolysis with a constant quantity of antiserum and a constant quantity of complement. When partial hemolysis greater or less than 50% was actually measured, the quantity for this endpoint was obtained by graphic interpolation from a plot of log dilution of antigen versus log (% hemolysis ÷ 100 — % hemolysis). The routine complement fixation test was set up in 8 tubes with dilutions of CRP test serum 1:10, 1:40, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200. The quantity of complement used was four 50% hemolytic units (about 0.005 ml undiluted guinea pig serum). Antiserum was used at a dilution of 1:40 (0.00125 ml). The standard CRP serum was run with each test at dilutions of 1:800, 1:1200, 1:1600, and 1:2400. Anticomplementary property of test sera at 1:10 dilution was examined with 2 units of complement.

Incidence of AC property (hemolysis of less than 55%) at this dilution is very low (less than 1%).

Microprecipitation reaction. This test was performed in capillary tubes(3) with the following modification to insure complete mixing of reagents. 0.01 ml of antiserum was placed in the well of a clear glass spot plate. 0.01 ml of test serum was added. The entire mixture was run into the capillary tube by tipping the plate. The contents of the capillary were then almost completely returned to the spot plate well by gentle (mouth) pressure. This procedure was repeated and the capillary tube was sealed after the third filling. Tubes were read by comparison against the standard CRP serum (equal to 160) at concentrations of 80, 40, 20, 10, 5 and 2½ after incubation (2 hours at 37°C, overnight at 4°C). With the antiserum used, the supernatant from the "80" tube produced no additional precipitate on adding either antiserum or CRP serum. The quantity of precipitate in the "2½" tube was less than 1 mm but more than a trace.

Gel diffusion. This test was carried out by the modification of the Ouchterlony technic described by Korngold and Lipari(7) which uses 1.5% concentration of agar. Plates were kept at room temperature for one week and then, as described by Libretti, *et al.*(5), at 4°C for 2 weeks.

Results. The complement fixation test for CRP involves 3 main variables—namely, the quantities of antiserum and complement which are required for the most effective measurement of a given quantity of CRP. The interrelationships are best studied by means of a block titration in which the quantity of complement is held constant and antiserum and antigen are varied. The data can be integrated into a line curve expressing the relationship between antigen and antibody over the full range of antibody excess, equivalence, and antigen excess by plotting the quantities of these reactants which always produce the same hemolytic endpoint(8). The relationship for the rabbit antibody-CRP system is shown in Fig. 1a. The curve is a non-rectangular hyperbola with asymptotes in the zones of antibody excess and antigen excess and a minimum close to the zone of equivalence. This curve is representative of systems in which the zone of antigen excess is characterized by marked inhibition of immune complex formation(8).

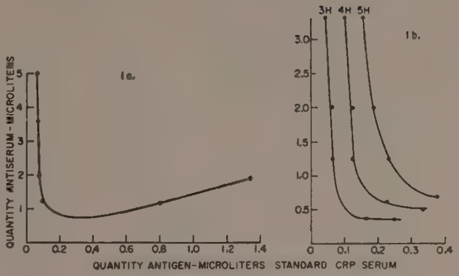

FIG. 1a. Antigen-antiserum reactivity curve for human CRP-rabbit antibody system.

FIG. 1b. Curves in zones of antibody excess and equivalence determined with 3, 4, and 5 hemolytic units of complement.

It can be seen that for the quantity of complement with which the curve is determined, a minimum quantity of CRP can be measured, no matter how large an excess of antibody is used. In Fig. 1b there is shown on expanded axes the zone of antibody excess determined with each of 3 different quantities of complement: 3, 4, and 5 units. These curves show how sensitivity of the determination (minimum quantity of measurable CRP) depends on quantity of complement used in the test, and further, how much antiserum must be used to insure antibody excess, once the sensitivity level has been selected. The choice of a particular set of quantities is a balance between supply of antiserum on one hand, and on the other, both quantity of CRP which must be determined and degree of interference from anticomplementary properties of the reactants.

A correlation of CRP titers determined for 192 specimens by both complement fixation and precipitation methods is shown in Fig. 2. It is evident that sensitivity of the method based on complement fixation has been set to conform exactly to sensitivity of that based on microprecipitation. Twenty-four specimens gave zero titers with both methods, 8 showed a CF titer of 1 without producing any precipitate, while 4 showed a precipitate corresponding to 1, 2 a precipitate correspond-

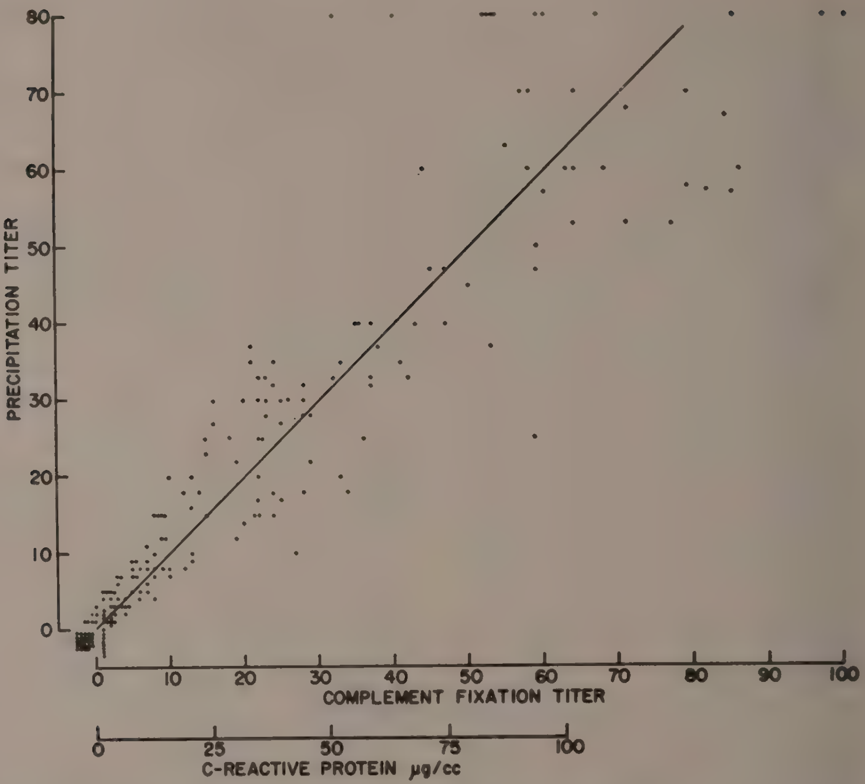

FIG. 2. Correlation of CRP levels determined by microprecipitation and complement fixation showing identical range of both methods. Specimens with very high complement fixation titers are not shown since maximum precipitation titer obtainable with undiluted test specimens is "80." Precipitation titers are the avg of observations by 3 individuals. The line represents the location of points for exact correspondence.

ing to 2, and 1 a precipitate corresponding to 3 without having any CRP determined by complement fixation. If we accept the greater reliability of the complement fixation method (since it is applied at a level considerably above the limit of its sensitivity), these results suggest that very small quantities of precipitate may not be specific for CRP. It has been frequently noted that traces of precipitate tend to redissolve on standing several minutes at room temperature. The relatively poor correlation between CRP titers obtained by the two methods is principally due to the error inherent in the subjective (visual) method of estimating the quantity of precipitate. Distinguishing a titer of "40" from one of "20" or "80" is frequently impossible, and the degree of correlation shown in Fig. 2 could be achieved only by averaging the estimates of 3 observers. The complement fixation method, on the other hand, offers an objective numerical index of the CRP titer. If 2-fold serial dilutions are tested and the exact dilution titer for 50% hemolysis is estimated by the graphical method described, an error of as much as 10% hemolysis produces an error of about 10% in the final result. Closer spacing of dilutions, as described for the standard, can appreciably decrease this source of variation.

The hemolytic unit of complement (quantity of guinea pig serum) which is defined by the indicator reaction serves as a control of the reagents participating in this part of the test. Fluctuations in the unit result principally from variations in batches of sensitized red cells and guinea pig serum. However, this index of variability in reagents is not applicable to the primary reaction. For this purpose, we have found that the use of a standard is essential(6). A convenient stable standard is a high-titered CRP serum specimen. The data in Table I representing dilution titers of the standard serum determined over a 5 month period and complement unit on the day of each determination show that the standard is stable. They also indicate that with a higher complement unit, a lower dilution titer is observed. This explanation is not sufficient to account for the variation or to eliminate the necessity for the standard, as is seen by inspecting the values on 1/11, 1/18, 1/27 and 1/31. On these days the complement unit was constant but the standard changed 35 to 45%.

The quantity of CRP in the standard was 280 μg/ml. Average titer was about 1500. Since 0.1 ml of antigen dilution is used, the endpoint in the complement fixation test requires about 0.02 μg of C-reactive protein. The sensitivity of the test described here (with limiting dilution of 1:10) therefore corresponds to 2 μg CRP/ml undiluted serum, a result in excellent agreement with the report of Wood and McCarty‡(3). An example of the calculation of CRP titer or concentration based on the standard is as follows. Assuming the test specimen shows a dilution titer of 350 when the standard serum dilution titer is 1500, then

$$\text{Test specimen CRP titer is } \frac{350}{1500} \times 160 = 37.$$

$$\text{Test specimen CRP conc. is } \frac{350}{1500} \times 280\ \mu\text{g/ml} = 65\ \mu\text{g/ml}.$$

In gel diffusion studies by the method of Ouchterlony we have not observed the multiplicity of CRP components reported by Libretti, *et al.*(5), either with 20 high titered specimens from different individuals or a pool of 20 such specimens. On prolonged standing (more than 10 days), a pattern similar to the one they describe was seen, but this is an artifact of the diffusion process, particularly in the region of antibody excess(9).

TABLE I. Titers of Standard CRP Serum and Complement over 5 Month Period Showing Stability of Standard and Relative Independence of Fixability and Hemolytic Activities of Guinea Pig Complement.

Date	Dilution titer of standard CRP serum	Complement unit, ml g.p. serum
9/12	1000	.00142
11/ 1	1800	.00122
11/ 9	1750	.00110
11/16	1450	.00110
11/30	1300	.00110
12/ 7	1750	.00121
12/14	1700	.00110
12/21	1150	.00121
12/28	1450	.00105
1/ 5	1400	.00100
1/11	1900	.00105
1/18	1350	.00105
1/27	1750	.00107
1/31	1300	.00105
2/ 8	1250	.00122

Discussion. In the procedure herein described, we have paid particular attention to 3 aspects of quantitative immunochemical analysis by complement fixation. These are the removal of residual anticomplementary activity from absorbed antisera, the necessity for a standard to eliminate variations as large as 2-fold in "fixability" of complement, and the selection of the most suitable quantity of antiserum for a given purpose. The range of C-reactive protein levels usually found in human serum is 0 to 300 μg per ml(3). The level can rise well above 300 μg after surgery (10). The large range taken in conjunction with the observed inhibition by excess antigen determines minimum number of tubes which must be set up for the complement fixation test. A 10 to 20-fold excess of antigen over the quantity measured at the endpoint is sufficient to produce hemolysis indicating a weak or negative test; the inhibition produced by a lesser degree of antigen excess may still appreciably affect determination of titer.‖ Suitable modifications of the complement fixation method will permit measurement of considerably lower concentrations of C-reactive protein than 2 μg per ml. However, as sensitivity is increased, two limitations become progressively more important: incidence of anticomplementary activity in test specimens, and C-reactive protein interaction with normal serum constituents which affects its reaction with antibody.

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‡ The values for concentration of CRP in Table 4 of this reference should be divided by 2 according to the authors.

‖ Since titer is usually determined from hemolysis greater or less than 50% by interpolation, a partial hemolysis in the zone of inhibition will incorrectly reduce the titer.

Summary. A quantitative immunochemical method for determination of C-reactive protein concentration in serum is described. The sensitivity of this method, based on complement fixation, has been selected to measure the same concentration range of CRP as determined by microprecipitation. Two important features generally applicable to complement fixation methods are presented; namely, removal of anticomplementary activity from absorbed antisera and use of a reference standard to compensate for variations in fixability of complement.

1. Tillet, W. S., and Francis, T., *J. Exp. Med.*, 1930, v52, 561.
2. Anderson, H. C., and McCarty, M., *Am. J. Med.*, 1950, v8, 445.
3. Wood, H. F., and McCarty, M., *J. Clin. Invest.*, 1951, v30, 616.
4. Muschel, L. H., and Weatherwax, R. J., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 191.
5. Libretti, A., Kaplan, M. A., and Goldin, M., *ibid.*, 1955, v90, 481.
6. Rapport, M. M., and Graf, L., *Cancer*, 1955, v8, 538.
7. Korngold, L., and Lipari, R., *Cancer Res.*, 1955, v15, 159.
8. Almeida, J. O., *J. Immunol.*, 1956, v76, 259.
9. Korngold, L., personal communication.
10. Rapport, M. M., Schwartz, A., and Graf, L., to be published.

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Pheochromocytomas of Adrenals in Male Rats Chronically Injected with Pituitary Growth Hormone.* (22667)

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Increased numbers of tumors of the types occurring spontaneously in the rat were observed during the course of chronic injection of normal female rats with pituitary growth hormone. Pulmonary and lymphatic tissues, reproductive organs, pituitary and adrenal glands were involved(1-6). Outstanding was the occurrence of adrenal medullary pheochromocytomas(2). Since none of this work had hitherto been done in male rats, the questions arose as to whether males would be equally responsive in body growth and similarly susceptible to tumor formation, especially as regards reproductive organs and adrenal glands.

Materials and methods. Male rats of the Long-Evans strain, of 2 different age groups, 6 and 14 months, were divided into experimental and control groups of 8 rats each. The purified growth hormone(7) was injected intraperitoneally in saline 6 days weekly. The initial daily dose of 0.5 mg was increased progressively during the first 160 days, reaching a maximum of 3.0 mg in the younger, and 3.5 mg in the older group. These doses were then maintained for the remainder of the 15-month injection period. Complete autopsies were performed on all rats. The weights of all organs were determined, and the entire organ or representative blocks were fixed and prepared for histological study.

Results. Body weight and length. The rats in the 6-month-old group weighed approximately 400 g at the beginning of the experiment and gained steadily, reaching a maximum of almost 700 g at 18 months (Fig.

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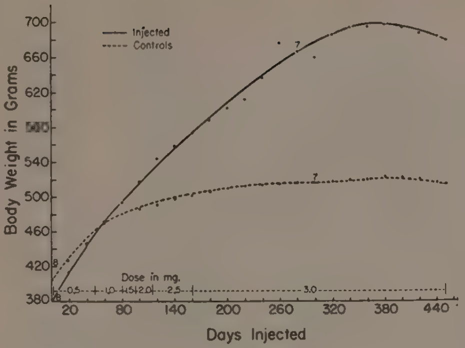

FIG. 1. Avg body wt of male rats inj. with growth hormone for 15 mo, beginning at 6 mo of age, compared with uninj. controls. Dosage shown in scale above abscissa. (No. of rats on which avg is based is indicated by numbers above curves.)

1). During the last 90 days there was cessation of growth and some loss of weight, despite the high daily dose. The rats of the 14-month group (Fig. 2) were heavier at onset (480 g) and their gain was slower and less regular, the maximum weight attained during the 15-month period being slightly less than that of the younger group, even though they received a higher dose. Both experimental groups increased in total length above their controls, and the greater increase occurred in the younger group.

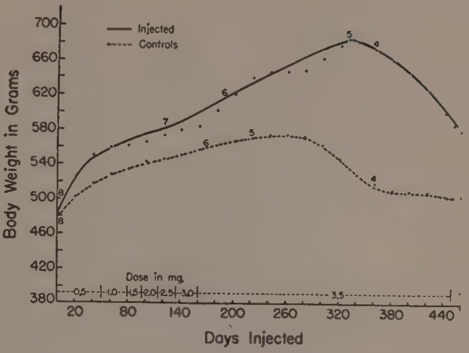

FIG. 2. Avg body wt of male rats inj. with growth hormone for 15 mo, beginning at 14 mo of age, compared with uninj. controls.

Blood pressure. Because of the anticipated development of pheochromocytoma, a neoplasm which had occurred frequently in female rats similarly treated(3), determinations of blood pressure were made at intervals during the course of this experiment. A microphonic manometer for determination of the systolic blood pressure was used(8). The animals were anesthetized with ether for determination of blood pressure, and body length was determined at the same time. No significant differences were observed in the blood pressure of experimental and control rats.

Neoplastic lesions. Neoplasms occurred in lymphoid tissue, adrenals, pituitary and thyroid glands. There was nothing significant in regard to the reproductive system. The number of tumors in the growth hormone injected rats of the 2 ages was not significantly different from that in the controls,† except in the tumors of the adrenal medulla. Neoplasms of the adrenal medulla were the most outstanding lesions observed in the experimental animals. Even in the adrenals where no pheochromocytomas were present, there was enlargement of the medullas. None of the controls of either age group had pheochromocytomas; 3 had focal areas in the medulla composed of uniformly small cells adjacent to blood vessels. Pheochromocytomas were

TABLE I. Neoplasms in Growth Hormone Injected Male Rats and Controls (8 per Group).

	Age at onset of exp.			
	6 mo		14 mo	
Type of neoplastic lesion	Inj.	Controls	Inj.	Controls
Pheochromocytoma	4	0	5*	0
Adrenal cortical adenoma	0	1	0	1
Thyroid: Adenoma	3	4	3	1
Carcinoma	1	0	1	1
Pituitary, adenoma	4	6	4	5
Testis, Leydig-cell tumor	1	0	0	0
Thymus, squamous cell carcinoma	0	1	0	0
Lymphosarcoma, thoracic and abdominal	1	0	4	3
Mesenchymal sarcoma (synovioma)	1	0	0	0
Kidney, papillary cystadenoma	0	0	1	0

* Bilateral in 4.

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† Numbers of tumors exclusive of pheochromocytomas, in the 6 months old group, 11 in experimental, 12 in control; in the 14 months old group, 13 in experimental, 11 in control.

present in 9 of the 16 rats treated with growth hormone; multicentric foci were present in some (Fig. 3-5). In the younger group, 4 of 8 rats showed unilateral tumors. In the older

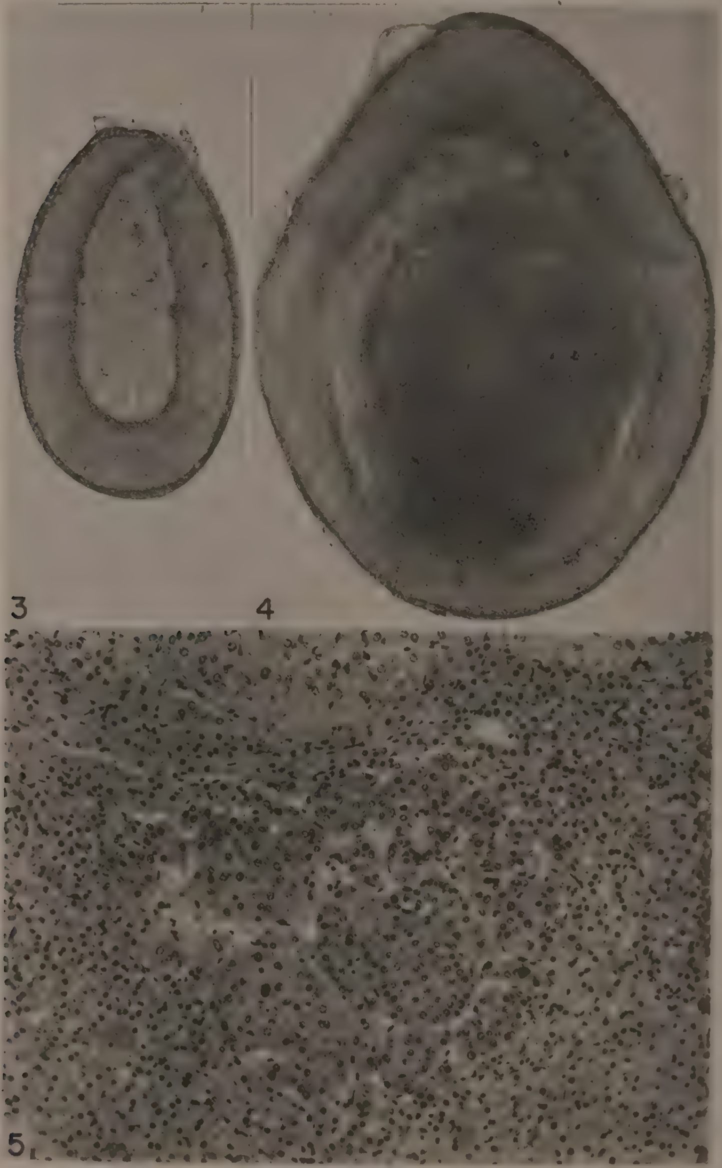

PLATE I (Fig. 3-5). Adrenals of adult male rats, control and exp.
(H and E stain)

FIG. 3. Control, untreated. Age 21 mo. × 22.

FIG. 4. Large medullary pheochromocytoma. Note compression of normal medullary tissue. Growth hormone inj. begun at 6 mo of age. × 22.

FIG. 5. Small pheochromocytoma composed of large cell types; surrounded by cells of zona reticularis. Growth hormone inj. begun at 14 mo of age. × 250.

group there were lesions in 5 of 8 rats and in 4 instances these were bilateral. Invasion of blood vessels by neoplastic cells, as described by Gillman *et al.*(9) in spontaneous pheochromocytomas of the rat, was not observed.

Discussion. Analysis of the results of chronic administration of pituitary growth hormone to adult male rats indicates that growth response was similar to, but less striking than, that of females so treated. Growth response of the older of the 2 age groups tested (14 months old at onset) was inferior to that of the younger group (6 months old at onset), particularly in the later part of the experiment. This may have been due to a number of factors, such as aging, debility due to neoplasms, and recurrent infections. In the earlier studies on females injected with growth hormone, pheochromocytomas frequently occurred. There was no evidence of a prolonged hypertension in those animals, as judged by weight of the heart and histology of the arterioles. In the present study no significant elevations of blood pressure were observed in experimental rats subsequently shown to have pheochromocytomas. However, the possibility of transient episodes of hypertension cannot be excluded.

Summary. The long term administration of pituitary growth hormone to adult male rats of the Long-Evans strain resulted in progressive growth in body weight and length. Many neoplasms developed in the rats during this prolonged period, but the incidence in treated rats was not significantly different from that in controls, with the exception of tumors of the adrenal medulla. Pheochromocytomas occurred in 9 of 16 growth hormone injected rats and in none of the controls.

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1. Evans, H. M., Simpson, M. E., and Li, C. H., *Growth,* 1948, v12, 15.

2. Moon, H. D., Simpson, M. E., Li, C. H., and Evans, H. M., *Cancer Research,* 1950, v10, 297.

3. ———, *ibid.,* 1950, v10, 364.

4. ———, *ibid.,* 1950, v10, 549.

5. Koneff, A. A., Moon, H. D., Simpson, M. E., Li, C. H., and Evans, H. M., *ibid.,* 1951, v11, 113.

6. Moon, H. D., Simpson, M. E., Li, C. H., and Evans, H. M., *ibid.,* 1951, v11, 535.

7. Li, C. H., Evans, H. M., and Simpson, M. E., *J. Biol. Chem.,* 1945, v159, 353.

8. Friedman, M., and Freed, S. C., Proc. Soc. Exp. Biol. and Med., 1949, v70, 670.

9. Gillman, J., Gilbert, C., and Spence, I., *Cancer,* 1953, v6, 494.

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Influence of Acetylcholine on Human Pulmonary Circulation under Normal and Hypoxic Conditions.* (22668)

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Short periods of induced hypoxia characteristically produce a moderate increase in the pressure in the pulmonary artery in normal man(1). In general, the rise in pressure is greater than might be expected to be due to the increase in cardiac output which occurs. Moreover, the possibility that the augmented pressure results from generalized systemic vasoconstriction seems unlikely, since the pulmonary wedge pressure does not change(2,3) the brachial artery pressure remains constant, and the "central blood volume" shows no consistent alteration(3,4). From these data it may be inferred that the

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rise in pulmonary artery pressure is probably produced by active constriction of the vessels of the lungs.

Previous experiments(5,6) have led us to believe that acetylcholine has a vasodilating action in the human pulmonary circulation. In diseased states there is evidence that this action is to some extent dependent on the preexisting tone in the vessels of the lungs(5,7). The effect of acetylcholine might, therefore, be expected to be more evident when the tone in these vessels has been increased by hypoxia.

The present study was undertaken to investigate the effect of acetylcholine on normal pulmonary circulation, and to determine whether the effect was modified by the presence of hypoxia. The purpose of this note is to report the results obtained in studies performed on 6 normal subjects.

Methods. The procedure combined catheterization of the pulmonary artery and cannulation of the brachial artery. Each subject breathed through a mouthpiece for 2 periods of 20 minutes separated by a rest of 15 minutes. During one period, the subject breathed ambient air and, during the other, a mixture of 12% oxygen in nitrogen. Between the 11th and 15th minute of each period, acetylcholine was infused through the catheter into the main pulmonary artery at a rate of 0.5 mg/min. Measurements of the pressure in the pulmonary and brachial arteries and of the cardiac output were made before and during the infusion of acetylcholine.

While the subject breathed ambient air, the cardiac output was estimated by the Fick principle. Under hypoxia, the Stewart-Hamilton dye-dilution technic was considered preferable since insufficient time was allowed for the establishment of the "steady state" required for the application of the direct Fick method. The dye output was obtained by injecting Evans' blue dye into the main pulmonary artery while inscribing a dilution curve from the brachial artery by means of a Colson densitometer.

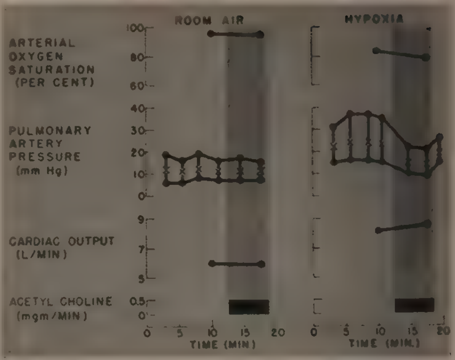

FIG. 1. Effect of acetylcholine on arterial oxygen saturation, pulmonary arterial pressure and cardiac output in one subject. The subject breathed room air for 20 min. and 12% oxygen for 20 min. Shaded areas represent time of infusion of acetylcholine. A period of 15 min. elapsed between the 2 experiments.

Results. Six satisfactory studies have been completed with similar results in each. The data from one of these are illustrated in Fig. 1. When the subjects breathed ambient air, acetylcholine caused a slight fall in the pulmonary arterial pressure. This response was much more evident after hypoxia had raised the pressure in the pulmonary artery. The reduction in pressure during the infusion of the drug occurred despite the fact that the cardiac output had either remained constant or had slightly increased. The infusion caused no alteration in the heart rate, the brachial arterial pressure, the arterial oxygen saturation, or the ventilation. In 2 subjects, pulmonary wedge pressures were recorded and found not to change during the infusion of acetylcholine. Thus, since the pressure in the pulmonary artery had fallen, the gradient of pressure was decreased.

Discussion. In these studies, the cardiac output remained unchanged or increased during the infusion of acetylcholine; it never decreased. Moreover, the constancy of the wedge pressure measured in 2 cases suggests that the left atrial pressure was unaffected by the drug. Therefore, it is believed that the fall in pulmonary arterial pressure resulted from an active dilatation of the pulmonary vessels. The augmented effect observed during hypoxia suggests that the action of the drug is dependent on the pre-existing tone of the pulmonary vessels.

Summary. Acetylcholine infused into the main pulmonary artery caused a slight fall in pulmonary arterial pressure when the subject

breathed ambient air, but a greater fall in pressure after pulmonary hypertension had been produced by hypoxia. The fall in pressure was associated with either a constant or an increased cardiac output. The evidence suggests that acetylcholine causes vasodilatation in the lungs and that this action is largely dependent on the pre-existing tone of the pulmonary vessels.

1. Motley, H. L., Cournand, A., Werkö, L., Himmelstein, A., and Dresdale, D., *Am. J. Physiol.*, 1947, v105, 315.

2. Westcott, R. N., Fowler, N. O., Scott, R. C., Hauenstein, V. D., and McGuire, J., *J. Clin. Invest.*, 1951, v30, 957.

3. Doyle, J. T., Wilson, J. S., and Warren, J. V., *Circulation*, 1952, v5, 263.

4. Fritts, H. W., Jr., Braunwald, E., Fishman, A. P., and Cournand, A., *Fed. Proc.*, 1956, v15, 68.

5. Harris, P., Thesis, Univ. of London, 1955.

6. Cournand, A., Fritts, H. W., Jr., Harris, P., and Himmelstein, A., *Tr. A. Am. Physicians*, in press.

7. Harris, P., *Brit. Heart J.*, 1955, v17, 85.

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Relationship of Bat Salivary Gland Virus to St. Louis Encephalitis Group of Viruses.* (22669)

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The existence of rabies in bats in the United States was first suspected in 1951 when a woman in Big Spring, Texas died of rabies 3 weeks after being bitten by a bat. This experience was reported(1) after the first demonstration of rabies in bats in the United States when the virus was demonstrated in a Florida yellow bat (*Dasypterus floridanus*) which had attacked a child(2). Three months after this incident another case was reported in Pennsylvania where, without provocation, a woman was bitten on the arm by a bat (probably *Lasciurus cinereus*) from which the rabies virus was isolated(3,4). These and other experiences prompted several groups to initiate studies to assess the importance of these animals as reservoirs and carriers of the infection(5-10). The rabies virus has now been recovered from bats in widely scattered areas in the United States (Florida (2), Pennsylvania(3), Texas(6,7), California (10), and Montana(11).)

During the course of their studies on rabies in nonsanguivorous bats in Texas, Burns and Farinacci(8,12,13) recovered a number of viral agents in addition to the rabies virus from the salivary glands of encephalitic bats. Following preliminary tests(13) which suggested that these agents were antigenically related to the St. Louis encephalitis virus, one strain was referred to us for determination of its antigenic relationship to the Japanese (JE)-West Nile (WN)-St. Louis (SLE)-Murray Valley (MVE) encephalitis group(14-19). This report is concerned with the relationship of the bat salivary gland (SG) virus to the JE-WN-SLE-MVE group as established by cross complement-fixation tests using hyperimmune guinea pig sera.

Viruses. The bat salivary gland virus (SG) designated #1410-19 and in its 5th mouse brain passage was sent to us by Lt. Col. Kenneth F. Burns. The Nakayama strain of JE virus in the 41st mouse brain passage, a strain of MVE in the 10th passage, and the WN virus (J7259) in the 25th passage were obtained from the Viral and Rickettsial Disease Registry, American Type Culture Collection, Washington, D.C. The Hubbard strain of SLE was obtained from Dr. Isaac Rushman.

Antigens and antisera. The antigens and the antisera for the complement fixation tests and the grid type test using these materials are essentially the same as described by Pond and his associates(19) and by Hammon and Espana(20). Antisera against the JE, WN, SLE and MVE viruses were obtained from

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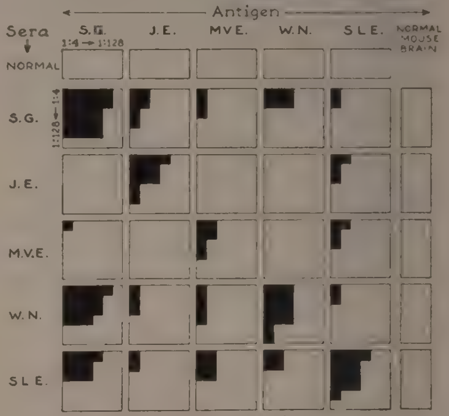
guinea pigs inoculated intracerebrally with suspensions of infected hamster brain. In each instance the virus contained in infected mouse brain tissue in 10% normal rabbit serum was passed intracerebrally through 3 serial passages in weaned hamsters. The guinea pigs were given 2 injections, 10 days apart, each consisting of 0.15 ml of a 10% suspension of 3rd passage infected hamster brain tissue. The sera obtained 2 weeks after the last injection were stored at —40°C. Each serum was inactivated at 56°C for 30 minutes before it was used in the complement fixation test. Since the SG virus is not infective for young hamsters, antiserum against this agent was prepared as described above, except 6th mouse brain passage material in 10% normal guinea pig serum in saline was used for immunizing the guinea pigs. A control group of guinea pigs each received two injections of 10% normal mouse brain in 10% guinea pig serum in saline.

In the initial tests it was found that all of the viruses except SG yielded effective complement-fixing antigens prepared by the technic of Havens *et al.*(21) or Casals *et al.*(22). Since highly effective antigens with the SG virus were obtained by the benzene extraction technic of DeBoer and Cox(23), all complement fixation tests were carried out with antigens prepared by this method.

Complement fixation tests. The complement fixation tests used in these studies are essentially the same as those described by Pond *et al.*(19). Serial 2-fold dilutions of each antigen were tested against serial 2-fold dilutions of each of the guinea pig antisera. In addition to the usual serum and antigen anti-complementary controls, normal guinea pig serum in serial dilution was tested against each dilution of the various antigens. In the test 2 exact units of complement were used. Following overnight incubation at 4°C the indicator system consisting of 3 units of hemolysin and 3% washed sheep cells was added. The tests were read after an additional incubation for 30 minutes at 37°C. Tubes with 50% or more fixation were considered positive.

RELATIONSHIP OF THE SALIVARY GLAND VIRUS OF BATS TO THE JE-MVE-WN-SLE GROUP ON THE BASIS OF COMPLEMENT FIXATION TESTS USING HYPERIMMUNE GUINEA PIG SERA



Shaded areas represent dilutions giving at least 50 % fixation.
Antigens and antisera were not anticomplementary at dilutions used.

FIG. 1.

Results. The results of a grid-type complement fixation test are presented graphically in Fig. 1 using the method previously described by Pond *et al.*(19). The shaded areas indicate mixtures in which 50% or more fixation of complement occurred. The results with the JE, MVE, WN and SLE antigens are in some respects the same as described by Pond *et al.*(19), although the higher titered SLE antigen used in these tests demonstrated serological relationships with other members of the family which were not evident in their study. For example, it can be seen that the JE antiserum reacted with its homologous antigen and to some extent with the SLE antigen, but failed to react with the MVE antigen. Pond *et al.*(19), however, found that JE antiserum reacted with the MVE antigen but not with the SLE antigen. In the results described in Fig. 1 it can be seen that the SLE antigen reacted to some extent with each of the antisera tested.

The results indicate that the SG agent appears to be a new member of the JE-WN-SLE-MVE group of viruses and that it is more closely related antigenically to WN than to other members of the group. These data, together with the fact that the SG antigen failed to fix complement in the presence of antisera prepared against a variety of other neurotropic viruses, confirm the preliminary observations of Burns and Farinacci(13). Additional studies are in progress to establish whether the serological relationships observed in the complement fixation test can be more effectively demonstrated by other immunologic technics.

Summary. A virus recently isolated from the salivary glands of Mexican free-tailed bats (*Tadarida mexicana*) appears to be a new member of the JE-WN-SLE-MVE group of viruses. On the basis of the results of complement fixation tests it appears that the salivary gland virus of bats is more closely related to the West Nile virus than to other members of the group.

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1. Sulkin, S. E., and Greve, M. J., *Texas State J. Med.*, 1954, v50, 620.
2. Venters, H. D., Hoffert, W. R., Scatterday, J. E., and Hardy, A. V., *Am. J. Pub. Health*, 1954, v44, 182.
3. Witte, E. J., *ibid.*, 1954, v44, 186.
4. Kough, R. H., *J. Am. Med. Assn.*, 1954, v155, 441.
5. Malaga-Alba, A., *Texas Health Bull.*, 1953, v6, 4.
6. Sullivan, T. D., Owens, J. E., Eads, R. B., Menzies, G. C., and Irons, J. V., *Pub. Health Rep.*, 1954, v69, 766.
7. Burns, K. F., and Farinacci, C. J., *Science*, 1954, v120, 548.
8. ———, *J. Inf. Dis.*, 1955, v97, 211.
9. Sulkin, S. E., Krutzsch, P. H., Wallis, C., and Allen, R., to be published.
10. Enright, J. B., Sadler, W. W., Moulton, J. E., and Constantine, D., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 94.
11. Bell, J. F., Hadlow, W. J., and Jellison, W. L., *Pub. Health Rep.*, 1955, v70, 991.
12. Burns, K. F., Farinacci, C. J., and Murnane, T. G., *J. Am. Vet. Med. Assn.*, 1956, v128, 27.
13. Burns, K. F., and Farinacci, C. J., *Science*, 1956, v123, 227.
14. Kudo, M., Uraguchi, K., Matusda, S., and Hoshimoto, H., *J. Immunol.*, 1937, v32, 129.
15. Smith, M. G., and Reames, H. R., PROC. SOC. EXP. BIOL. AND MED., 1940, v45, 838.
16. Smithburn, K. C., *J. Immunol.*, 1942, v44, 25.
17. Casals, J., *J. Exp. Med.*, 1944, v79, 341.
18. Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1946, v52, 235.
19. Pond, E. L., Russ, S. B., Rogers, N. G., and Smadel, J. E., *ibid.*, 1955, v75, 78.
20. Hammon, W. McD., and Espana, C., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 113.
21. Havens, W. P., Watson, D. W., Green, R. H., Lavin, G. I., and Smadel, J. F., *J. Exp. Med.*, 1943, v77, 139.
22. Casals, J., Olitsky, P. K., and Onslow, R. O., *ibid.*, 1951, v94, 123.
23. DeBoer, C. J., and Cox, H. R., *J. Immunol.*, 1947, v55, 193.

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Choline Antimetabolites. Studies with Intestinal Muscle. (22670)

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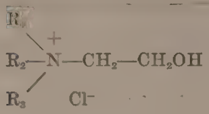
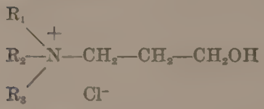
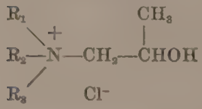
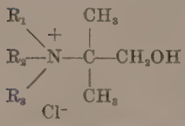
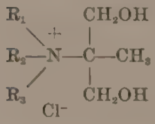
The report of Keston and Wortis(1) that frog muscle contracts in response to choline and that this contraction is inhibited by triethylcholine was of interest to us in our studies of compounds structurally similar to choline. Triethylcholine is only a very weak inhibitor of choline oxidation by choline dehydrogenase(2) and has usually been found to have choline-like activity (*Neurospora crassa* growth(3); lipotropic(4); antihemorrhagic (5)). In view of this anomalous behavior of triethylcholine with muscle we have extended our studies to include the effects of choline and related compounds on muscle contraction.

In this study segments of rabbit ileum were used instead of frog muscle. This acetylcholine sensitive tissue contracts in response to choline. We found that the response to choline was inhibited competitively by triethylcholine, β-methyltriethylcholine and α-methyl-α-hydroxymethylcholine. Triethylcholine was not, however, an inhibitor of acetylcholine. Methyldiethylcholine, triethylhomocholine, and α-methyl-α-hydroxymethyltrie-

TABLE I. Effect of Choline Analogues on Rabbit Ileum Muscle.

Compounds tested	R_1	R_2	R_3	Choline-like (relative activity, choline = 1.0)	Anti-choline
 $\mathrm{R_1R_2R_3\overset{+}{N}{-}CH_2{-}CH_2OH\ Cl^-}$	H	H	H	.3*	
	E	E	H	.1	
	M	M	E	4.0	
	M	E	E	.0	—
Ethanolamine	E	E	E	.0	+
 $\mathrm{R_1R_2R_3\overset{+}{N}{-}CH_2{-}CH_2{-}CH_2OH\ Cl^-}$	H	H	H	.2	
	E	E	H	.1*	
	E	E	E	.0	—
3-Aminopropanol-1	M	E	E	.1	
 $\mathrm{R_1R_2R_3\overset{+}{N}{-}CH_2{-}\overset{CH_3}{\overset{\vert}{C}}HOH\ Cl^-}$	E	E	H	.1*	
	M	E	E	.1*	
	M	M	M	.5	
1-Aminopropanol-2	E	E	E	.0	+
 $\mathrm{R_1R_2R_3\overset{+}{N}{-}C(CH_3)_2{-}CH_2OH\ Cl^-}$	H	H	H	.1*	
	M	M	M	.2	
2-Amino-2-methylpropanol-1	E	E	E	.1	
 $\mathrm{R_1R_2R_3\overset{+}{N}{-}C(CH_2OH)_2{-}CH_3\ Cl^-}$	H	H	H	.1*	
	M	M	M	.0	+
2-Amino-2-methylpropanediol-1,3	E	E	E	.0	—
Thiocholine chloride				3.1	

* Transient response. M = Methyl; E = Ethyl.

thylcholine were inactive at the concentrations tested while the remaining compounds studied possessed various degrees of choline-like activity.

Methods and materials. The choline analogues were prepared by methods previously described(2). Rabbit ileum was obtained soon after sacrifice of the animal, washed with Tyrode solution and then immersed in this medium at 5°C until used. A small segment was suspended in 100 ml of Tyrode solution at 37°, through which was bubbled a mixture of 95% O_2 + 5% CO_2, and its contractions recorded with a frontal writing lever on a kymograph.

When the spontaneous contractions had become constant, 2 mg of choline chloride were added and the increased contractions recorded. The bathing medium was then removed; the segment was washed 3 times with 50 ml of fresh Tyrode solution (37°), and resuspended in Tyrode solution as above. These operations could be repeated several times with essentially the same response to choline; the response to choline chloride was proportional to the dose up to a level of 6 mg.

The effects of the choline analogues on the segments of ileum were determined by adding the former to the bathing medium 2 mg at a time until 6 mg had been used. The testing of each compound was preceded by a test with choline, which was then washed out as above, and the segment was discarded when its response to choline was appreciably decreased.

Those compounds which failed to cause an increase in the contraction of the segment were tested for anticholine activity. Two mg of choline chloride were added to the bathing medium, and after the increase in contraction was established, 6 mg of the test compound were added. If no change in the contractions was produced, the test compound was considered to have neither choline-like nor anticholine activity.

Results. The activities of the compounds tested are presented in Table I. Fourteen of the compounds produced a response like that given by choline; the potencies were usually less than 0.5 that of choline on a weight basis with the exception of dimethylethylcholine and thiocholine which were 4.0 and 3.1 times as active as choline respectively. Diethylmethylcholine, triethylhomocholine and α-methyl- α-hydroxymethyltriethylcholine were inactive at the concentrations tested while triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine possessed anticholine activities.

That the compounds having anticholine activities were actually competitive inhibitors of choline was established by determining response of the segments of ileum to choline in the presence of 15 mg of the inhibitory compounds. The results are plotted in Fig. 1. In each case the response curve was roughly parallel to that obtained with choline alone and displaced toward higher choline concentrations. Under the same conditions, however, these compounds were found to have choline-like activities with segments of jejunum.

Discussion. The ability of choline to cause contraction of intestinal muscle may be (a) an inherent property of the compound or such activity may result from (b) its conversion to acetylcholine or (c) its being an inhibitor of choline esterase. Whatever the mechanism of action is, it is reasonable to assume that the remaining compounds having choline-like activity would exert their activities by a similar means. That choline is a competitive inhibitor of choline esterase has been demonstrated (6,7). However, this does not seem to be the mechanism involved here since the anticholine effects of triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine would not then be explicable.

An unequivocal differentiation between the first two possible modes of action cannot be made from the experiments done in this study. However, there is some evidence against the second interpretation. First, we ob-

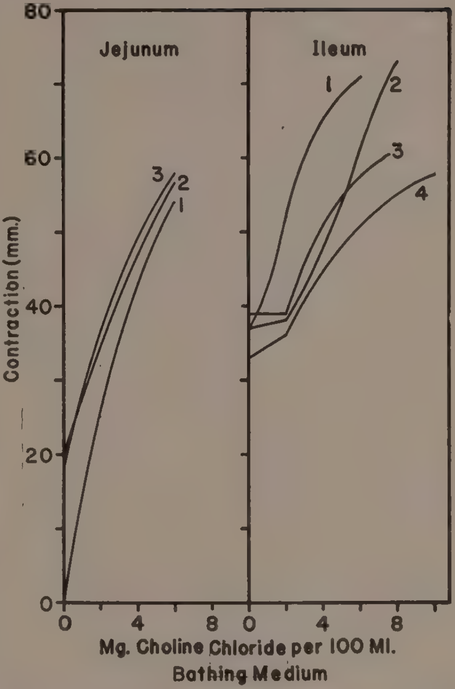


FIG. 1. Curve 1, choline alone; curve 2, choline plus 15 mg triethylcholine chloride; curve 3, choline plus 15 mg β-methyltriethylcholine chloride; curve 4, choline plus 15 mg α-methyl-α-hydroxymethylcholine chloride. In the segments of jejunum the contraction recorded was largely due to a change in tone. The contractions plotted for both tissues represent the sum of actual contraction and increased tone. Curves plotted are avg obtained with the same intestinal segment. Pieces of intestine from other animals gave similar results.

served that the contractions of the ileum began almost immediately on the addition of the choline and were constant after a relatively few contractions. If it were necessary to acetylate the choline in order for it to be active one might expect a delay followed by a relatively long period of increasing contractions. Second, by analogy to studies of the action of choline and related compounds on the heart of *Venus mercenaria*(8, 9) it seems also that the first possibility is more likely. It thus appears that our results are best explained by assuming that choline and certain other compounds related to it are capable *per se* of stimulating intestinal muscle. The mechanism of this action may be similar to the coenzyme theory proposed by Welsh(10) to explain the mode of action of acetylcholine. Accordingly, triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine have anticholine activity because they compete with choline for active sites on the apoenzyme.

Summary. Choline-like and anticholine activities of a series of compounds structurally related to choline have been determined using segments of rabbit ileum. Triethylcholine, β-methyltriethylcholine and α-methyl- α-hydroxymethylcholine were competitive inhibitors of choline in this tissue but had choline-like activity on segments of jejunum.

1. Keston, A. S., and Wortis, S. B., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 439.

2. Wells, I. C., *J. Biol. Chem.*, 1954, v207, 575.

3. Horowitz, N. H., Bonner, D., and Houlahan, M. B., *ibid.*, 1945, v159, 145.

4. Channon, H. J., and Smith, J. A. B., *Biochem. J.*, 1936, v30, 115.

5. Welch, A. D., *J. Nutr.*, 1950, v40, 113.

6. Roepke, M. H., *J. Pharmacol. Exp. Therap.*, 1937, v59, 264.

7. Ziff, M., Jahn, F. P., and Renshaw, R. R., *J. Am. Chem. Soc.*, 1938, v60, 178.

8. Welsh, J. H., and Taub, R., *Biol. Bull.*, 1948, v95, 346.

9. —— –, *J. Pharmacol. Exp. Therap.*, 1950, v99, 334.

10. Welsh, J. H., *Bull. Johns Hopkins Hosp.*, 1948, v83, 568.

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Effect of Aspirin Administration on Serum Glutamic Oxaloacetic and Glutamic Pyruvic Transaminases in Children.* (22671)

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(Introduced by H. F. Wood)
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Elevations of the serum glutamic oxaloacetic transaminase (SGO-T) activity during the course of rheumatic fever in children have been noted previously(1). Despite the irregular pattern of these elevations and the absence of parallel alterations in the indices of inflammation commonly used (erythrocyte sedimentation rate, white blood cell count and C-reactive protein) and in the clinical course of the patients, it was suggested that the alterations of SGO-T were the result of myocardial necrosis during the course of rheumatic carditis. This enzyme is known to be present in the heart in high concentration(2) and elevation of its activity in the serum following myocardial infarction in man(3) and in dogs (4) has been described. Elevations of SGO-T also occur in human(5) and murine(6) hepatic diseases, but no clinical evidence of hepatic disease was present in the rheumatic fever patients studied and their liver function tests were normal(1). Drugs such as digitalis and mercurial diuretics which are often used in the treatment of patients with rheumatic fever were shown to have no effect on

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† Research Fellow of the Arthritis and Rheumatism Foundation.

the SGO-T(3). Moreover, no alteration of SGO-T was noted during the administration of 4 to 6 g of aspirin daily for one week to a group of non-rheumatic adults(1).

Despite this latter observation a possible effect of aspirin upon the serum activity of glutamic oxaloacetic transaminase in children was suspected as a larger series of children with rheumatic fever was subsequently studied. The present investigation was undertaken to explore this relationship. The activity of another enzyme, glutamic pyruvic transaminase (SGP-T) was studied in the sera of a group of patients in an attempt to determine whether serum activity of another transaminase in addition to SGO-T was affected by aspirin administration. In addition, since glutamic pyruvic transaminase is found in the liver in 6 times the concentration found in the heart(7), it was hoped that the source of the increased serum concentration of SGO-T might be suggested by the results of simultaneous determinations of the two transaminases. It has, in fact, been reported, in contrast to the findings with SGO-T, that the serum activity of glutamic pyruvic transaminase (SGP-T) is increased only slightly following myocardial necrosis in man. Similar elevations of the serum activities of both enzymes are found in liver diseases(7).

Materials and methods. Twenty-three children from the wards of Irvington House, a hospital and convalescent home for children with rheumatic fever, were studied. Their ages ranged from 5 to 15.

Group 1: Of the 14 children included in this group 12 received aspirin (acetyl salicylic acid) and 2 sodium salicylate. None was considered to have definite evidence of active rheumatic fever: 7 had questionable disease activity and 7 had no evidence of rheumatic activity by all clinical and laboratory criteria. Aspirin and sodium salicylate were administered in daily dosages of 0.6 to 1 g per 15 lb of body weight for a minimum of 4 weeks. The SGO-T was measured during a short control period and then twice weekly during the period of salicylate administration.

Group 2: The second group selected for study of both SGO-T and SGP-T consisted of 9 children, 7 of whom had no clinical or laboratory evidence of rheumatic activity. One patient was studied during a mild clinical rebound of rheumatic fever. The ninth patient had acute rheumatic polyarthritis with no evidence of carditis. Aspirin was administered in a daily dose of 0.6 g per 15 lb of body weight for an average period of 16 days. The dose was then increased to 1 g per 15 lb of body weight until symptoms and signs of toxicity occurred. At this time the aspirin was discontinued.

The SGO-T and SGP-T activities were determined twice weekly during a short control period and during the period of aspirin administration. The specimens of blood obtained were allowed to clot at room temperature. The sera were then separated immediately and frozen and the determinations of enzyme activity were performed within 3 days. Care was exercised to prevent hemolysis since this has been shown to increase the measured SGO-T activity(8). SGO-T activity was measured by the spectrophotometric method of Karmen *et al.*(8). SGP-T activity was measured by a similar method described by Wróblewski and LaDue(7). By these methods the normal range of activity of SGO-T is 10-40 u/ml/min. and of SGP-T is 5-35 u/ml/min. Rises of SGO-T to 50 units or more and of SGP-T to 45 units or higher are considered to be significant.

Blood salicylate levels were determined twice weekly using the method of Brodie, Udenfriend and Coburn(9). C-reactive protein was determined weekly by the capillary precipitin method described by Anderson and McCarty(10). Erythrocyte sedimentation rate was determined weekly by the Wintrobe method(11). To rule out the possibility of a direct effect of aspirin upon the rate of change of optical density of the reaction mixture in the spectrophotometric methods used, 2 series of experiments were done: A. Solutions of aspirin in phosphate buffer (pH 7.4) were added to the reduced coenzyme I to final concentrations of 10 to 60 mg % and the optical densities of the solutions measured for 10 minutes. B. Both sodium salicylate and aspirin were dissolved in normal serum to

TABLE I. Effect of Aspirin and Sodium Salicylate Administration on Serum Glutamic Oxaloacetic Transaminase Activity.

Patient	Rheumatic activity	Drug administered	Maximal level of SGO-T (u/ml)
1	?	Aspirin	146
2	?	"	88
3	?	"	128
4	No	"	93
5	?	"	<50
6	No	"	132
7	"	"	70
8	"	"	<50
9	"	"	"
10	?	"	"
11	?	"	"
12	No	"	"
13	"	Sodium salicylate	100
14	?	" "	<50

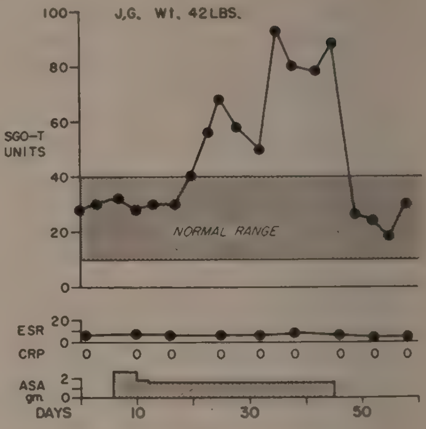

FIG. 1.

final concentrations of 5 to 40 mg %. The SGO-T and SGP-T activities of the sera were measured and compared with those of the same sera before the addition of these drugs.

Results. The effects of the administration of aspirin and sodium salicylate on serum transaminase activities in the patients of the 2 groups studied are summarized in Tables I and II. All patients had normal enzyme activities in their sera in the control period, before the administration of aspirin or of sodium salicylate. It is apparent that of 12 children in the first group receiving aspirin, 6 showed elevations of SGO-T to 50 units or greater. The maximal rise was to a level of 146 units. Three of these patients had no evidence of rheumatic activity and 3 were considered to have possible activity.

TABLE II. Effect of Aspirin Administration on Serum Glutamic Oxaloacetic and Serum Glutamic Pyruvic Transaminases.

Patient	Rheumatic activity	No. of days of aspirin admin. previous to 1st significant rise SGO-T	SGP-T	Maximal level SGO-T	SGP-T
1*	No	7	11	540	900
2	"	10	14	180	140
3	"	13	13	120	216
4	"	no rise	no rise	<50	<45
5	"	13	13	62	50
6	"	22	22	64	45
7	"	no rise	no rise	<50	<45
8	?	"	"	<50	<45
9	Yes	6	6	74	76

* This patient suffered from infectious hepatitis 18 mo earlier.

One of the 2 children receiving sodium salicylate showed an elevation of the SGO-T to 100 units. This patient did not have active rheumatic fever. The concentration of SGO-T in the other patient rose to 44 units, a borderline value.

A typical case is illustrated in Fig. 1. J.G., a patient convalescent from rheumatic fever and without evidence of rheumatic activity was maintained on aspirin, 1.2 to 2.4 g daily for more than one month. The SGO-transaminase first rose to an abnormal concentration 2 weeks after initiation of therapy, reaching a peak of 93 units 26 days after the first dose of aspirin. The SGO-T returned to a normal value within 2 days after discontinuation of the drug.

Table II shows the simultaneous variations of both SGO-T and SGP-T in the second group of 9 children in whom aspirin was administered until the appearance of toxic symptoms. Six of the children developed elevations of both SGO-T and SGP-T. Maximal levels of 540 SGO-T units and 900 SGP-T units were noted. These occurred in a patient who had had infectious hepatitis 18 months earlier. The shortest duration of aspirin administration resulting in significant rises of either enzyme was 6 days. Twenty-two days of continuous administration elapsed before the first elevation in patient No. 6, whereas patient No. 7 received aspirin for 32 days with no detectable rise in the serum activity of either transaminase, despite comparable dosages and serum salicylate levels. A second transitory rise of one or both enzymes was noted in 4 cases 3 to 10 days after cessation of aspirin therapy at a time when there was no detectable salicylate in the patients' sera (Fig. 2).

No consistent relationship between serum salicylate levels and serum enzyme activities was found. There was no evident correlation between changes in the indices of inflammation (erythrocyte sedimentation rate, white blood cell count and C-reactive protein) and SGO-T or SGP-T.

No change in the rate of the transamination reaction of the test solutions or of the optical density of reduced coenzyme I was produced by the *in vitro* addition of aspirin or of sodium salicylate. The concentrations of salicylate tested encompassed the range of concentrations measured in the sera of the patients studied.

Discussion. It is clear that aspirin administration can influence the serum activity of glutamic oxaloacetic and glutamic pyruvic transaminases in children. For this reason any increase in the serum activity of these enzymes during the course of rheumatic fever should be evaluated with great care when aspirin is employed in the therapy of these patients.

The mechanisms whereby this aspirin effect is mediated are unknown at the present time. A direct effect of aspirin upon the rate of the transamination reaction in the test mixture or upon the optical density of reduced coenzyme I is ruled out by the *in vitro* studies mentioned. Among the several other possible explanations the possibility of an increased release of these enzymes from the liver as a result of aspirin administration deserves consideration. Experimental and clinical studies have documented changes in hepatic function and morphology following aspirin administration(12). SGO-T has been shown to be a sensitive and early indicator of hepatic damage(5). Furthermore, the simultaneous elevation of SGO-T and SGP-T is consistent with the possibility that the liver is the source of the increase of the serum activity of these enzymes.

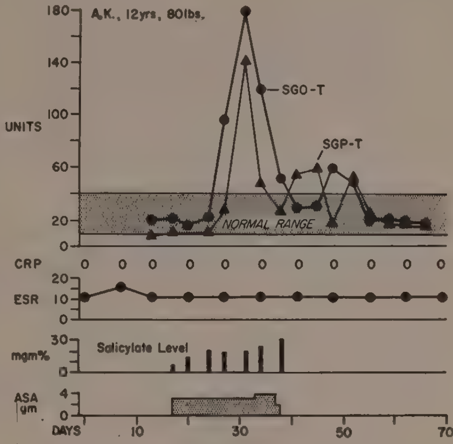

FIG. 2.

Summary. The effect of aspirin and of sodium salicylate administration on the serum glutamic oxaloacetic transaminase activity in 14 children and its effect on both the serum glutamic oxaloacetic transaminase and the serum glutamic pyruvic transaminase in an additional 9 children has been studied. Elevations to abnormal levels were observed in more than 50% of the cases. The importance of this finding in relation to the interpretation of the serum glutamic oxaloacetic transaminase elevations in rheumatic fever is pointed out and some possible underlying reasons for this phenomenon are mentioned.

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1. Nydick, I., Tang, J., Stollerman, G. H., Wróblewski, F., and LaDue, J. S., *Circ.*, 1955, v12, 795.

2. Cohen, P. P., *J. Biol. Chem.*, 1940, v140, 711.

3. LaDue, J. S., and Wróblewski, F., *Circ.*, 1955, v12, 161.

4. Nydick, I., Wróblewski, F., and LaDue, J. S., *ibid.*, 1955, v12, 161.

5. Wróblewski, F., and LaDue, J. S., *Ann. Int. Med.*, 1955, v43, 345.

6. Friend, C., Wróblewski, F., and LaDue, J. S., *J. Exp. Med.*, 1955, v102, 699.

7. Wróblewski, F., and LaDue, J. S., Proc. Soc. Exp. Biol. and Med., 1956, v91, 569.

8. Karmen, A., Wróblewski, F., and LaDue, J. S., *J. Clin. Invest.*, 1955, v34, 126.

9. Brodie, B. B., Udenfriend, S., and Coburn, A. F.,

J. Pharm. and Exp. Ther., 1944, v80, 114.

10. Anderson, H. C., and McCarty, M., *Am. J. Med.*, 1950, v8, 445.

11. Wintrobe, M., *Clinical Hematology*, 2nd Ed., 1946, p230.

12. Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics*, 1955, 2nd Ed., p286. Lutwack-Mann, C., *Biochem. J.*, 1942, v36, 706; Ritz, N. D., Samuels, L. T., and Adalis, G., *J. Pharm. and Exp. Ther.*, 1940, v70, 362; Troll, M. M., and Menten, M. L., *Am. J. Dis. Child.*, 1945, v69, 37.

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Corn Oil and Hypercholesteremic Response in the Cholesterol-Fed Chick.* (22672)

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Recently Ahrens(1,2), Kinsell(3,4,5,6) and Bronte-Stewart(7) and their coworkers have reported unusual reductions of the serum cholesterol in subjects receiving a formula diet in which various vegetable oils, in rather large quantities, were the main source of fat. Ahrens, *et al.*,(2) demonstrated that corn oil was the most effective of these agents in maintaining a low level of serum cholesterol. They also have shown a correlation between the iodine number and the hypocholesteremic effect of several vegetable oils, and have postulated that the degree of unsaturation of the fatty acids is responsible for that effect. Meanwhile, we had noted a lower level of serum cholesterol in cholesterol-fed control chicks used to assay fractions of a hypocholesteremic brain extract(8). This difference could be related to the fact that we had begun to use corn oil instead of cottonseed oil as a vehicle for the administration of cholesterol in the atherogenic diet.

The following experiments were performed in an effort to test the hypothesis that it is the slightly higher level of unsaturated fatty acids in corn oil which distinguishes it from cottonseed oil in its effect on the cholesterol-fed bird.

Methods. In each experiment, 40 8-week-old White Rock cockerels were divided into 4 dietary groups, each of which were offered equal weights of the diets. Diet consumption was estimated daily, body weight weekly. Two birds losing weight and apparently ill were excluded from the data. All of the diet offered was eaten, except as noted below. The chicks were bled from the alar vein at 2-week intervals, and the plasma analyzed for serum cholesterol by the method of Abell, *et al.*(9). At termination of the experiments, the aortae were examined for gross atherosclerotic plaques and graded as described by Horlick and Katz(10).

Experiment 1: To test the importance of the relative proportions of oleic and linoleic acids as they occur in corn oil as opposed to cottonseed oil, we used 2 "neo-fat" fatty acid products of Armour Chemical Division. Neo-Fat 105 consists of fatty acids redistilled from the cottonseed oil; Neo-Fat 110 is also derived from cottonseed oil fatty acids but fractionated so as to approximate the same relative proportions of palmitic, oleic and linoleic acids as occur in corn oil. According to the producers, these 2 distillates had the same minor difference in iodine number seen between corn oil and cottonseed oil, but neither had any myristic acid, which is normally present in corn oil. Each contained approximately 2% unsaponifiable matter derived from cottonseed oil. One group was fed 10% solvent extracted corn oil (Mazola); the second group 10% cottonseed oil (Puritan Oil, Procter and Gam-

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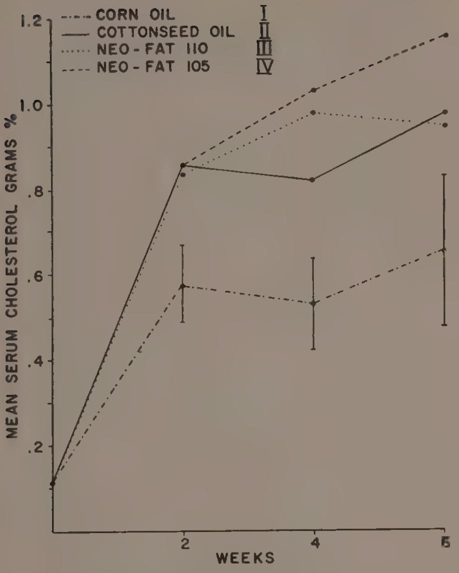

FIG. 1. Mean serum cholesterol in the 4 dietary groups indicated above. Vertical lines indicate ± one stand. dev. in the corn oil fed birds.

ble). The third group was fed 10% Neo-Fat #105, the fourth, 10% Neo-Fat #110 plus 0.1% myristic acid; each diet contained 1% cholesterol and Kay Bee chick starter mash. The latter contains ground corn, oats and wheat bran, plus alfalfa and soy bean meals which provide no less than 3% fat.

Experiment 2: Group I was fed 17.8% whole corn germ (containing 56% lipid material); Group II was fed 10% crude corn oil, expressed mechanically from the whole germ; Group III was fed solvent (hexane) extracted corn oil; and Group IV received 8% solvent extracted germ plus 9.8% cottonseed oil.‡ Thus each group received 10% lipid material by weight. One percent cholesterol was added to all diets, mixed in the oil or, in the case of whole germ, added in crystalline form. Stammler *et al.*(11) have shown that the mode of addition of cholesterol to the diet is not crucial. Each diet was mixed for at least 20 minutes in a Hobart mixer.

Results. The results of the first experiment may be seen in Fig. 1. The birds fed corn oil had a consistently lower serum cholesterol level than those fed cottonseed oil or either of its fatty acid distillates. This difference was statistically significant ($P<0.01$) at each of the 3 bleedings. No statistical significance could be found between mean plasma cholesterol levels at any bleeding of the birds fed cottonseed oil, as compared with its 2 distilled fatty acid products, though both of the distillation products tended to be more conducive of hypercholesteremia under these conditions than the original cottonseed oil. No significant difference in incidence of atherosclerosis was observed though the average grade of severity tended to correspond with the mean level of serum cholesterol.

The second experiment was devised to investigate a cruder preparation of corn oil and the whole corn germ. The cholesterol levels for the 4 groups are plotted in Fig. 2. The whole germ fed birds were significantly lower than all other groups at each bleeding, manifesting a very minimal elevation of the cholesterol level. Statistically, the level of probability is less than 0.01% in the last 2 bleed-

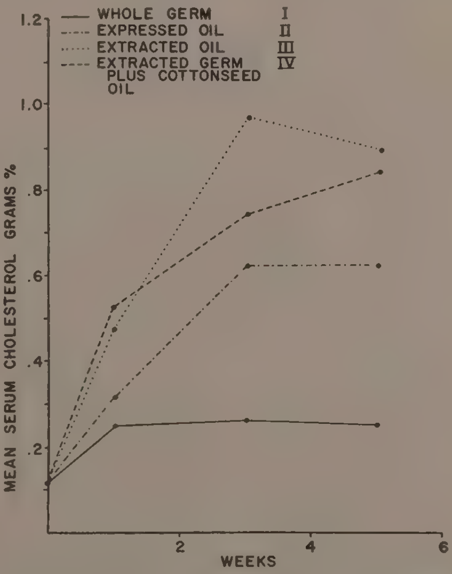

FIG. 2. Mean serum cholesterol in the 4 dietary groups indicated.

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‡ We are grateful to Dr. Artz of Corn Products Refining Co., Chemical Division, Argo, Ill. who supplied us with these preparations and their specifications.

TABLE I. Estimated Proportions of Dietary Foodstuffs and Degree of Hypercholesteremia and Atherosclerosis Observed Terminally in the 4 Dietary Groups.

Source of added fat	Grams/100 g of diet estimated			Terminal mean plasma cholesterol ± S.E.	Terminal mean atherosclerosis, aorta-grade (+ to 4+)
	Fat	CHO	Protein		
I. 17.8% whole corn germ	12.4	42.0	18.0	251 ± 29.1	.2
II. 10% crude expressed corn oil	13.0	44.5	17.8	632 ± 98.5	.6
III. 10% solvent extracted corn oil	13.0	44.5	17.8	905 ± 127	1.0
IV. 8% solvent extracted corn germ; 9.8% cottonseed oil	13.4	45.3	18.2	850 ± 112	1.0

ings. The cholesterol level is significantly lower in the expressed oil fed group than in the extracted oil group at the one and the 3-week bleedings ($P<0.05$), but not at the terminal bleeding. In this experiment the birds in Group III ate only 96%, and in Group IV, 88% of the diet offered. Groups I and II ate the full 100 g allowed per bird per day. It is doubtful that the difference between Groups II and III could be explained by the difference in cholesterol intake, though this may well explain why Group IV did not develop still higher levels of plasma cholesterol. The serum cholesterol levels achieved here are higher with corn oil than in the first experiment because of a greater dietary allowance of 100 g/day of the respective diets as opposed to 75 g/day per bird.

The results of grading the aortae of all birds in this experiment are shown in Table I. It can be seen, as with the plasma cholesterol levels, that there is a difference of borderline significance between the oil-fed groups; but the group getting whole germ is significantly protected against the early lesions seen in the other groups.

Discussion. Ahrens and coworkers(2) were able to correlate the iodine number roughly with the serum cholesterol-lowering effect, but other possible factors were not investigated. Certainly the data of Bronte-Stewart and his coworkers(7) confirms and more strongly suggests the importance of saturation of the fatty acids for the hypercholesteremic response to cholesterol feeding in the human. Swell and Flick(12) compared the response of the serum cholesterol in rats to cholesterol feeding in conjunction with lard, oleic acid or stearic acid, and concluded that the absorption of cholesterol appears to be decreased with a highly saturated fat. Later Swell and coworkers(13) showed substantial differences in the degree of elevation of the serum cholesterol levels of rats fed equal amounts of various fat sources. Here they found the response to be greatest in the descending order: Oleic acid, stearic acid, linoleic acid, peanut oil, corn oil and linseed oil. They also demonstrated that *in vitro* esterification of cholesterol proceeded more rapidly with fatty acids than with neutral fat.

These experiments leave little doubt that the serum cholesterol level is greatly influenced by the nature of dietary fat employed. They also reach fairly general agreement that corn oil seems to promote cholesterol absorption less than many other fats or fatty acid combinations, though Lin and coworkers(14) suggested that tripalmitin or other poorly utilized fats might be used even more profitably to effect a lower serum cholesterol. Our first experiment demonstrates that corn oil does not permit as great a hypercholesteremia as cottonseed oil, and further that this difference cannot be explained by the slight differences in fatty acid composition between these two oils. While there is nothing conclusive about our second experiment, it too suggests that the degree of saturation of the fatty acids is not the sole factor in determining the response to cholesterol feeding. Virtually all of the neutral fat is obtained from the corn germ by these two methods of extraction and it is doubtful that the fatty acids occur in any different proportions in the residue than in the expressed oil. The waxes and the unsaponifiable fraction are more apt to be left behind. It is doubtful that sitosterols could explain the effect, for they occur as only approximately one percent of the oil, (or 0.1

g per chick per day).

The various oils and corn germ products were added to the mash so that the fat level was essentially equal in each group, and it can be seen from Table I that protein and carbohydrate also remained in about the same proportion. Portman, *et al.*(15) have shown that the quality of dietary carbohydrate can be important in determining the hypercholesteremic response in cholesterol-fed rats. They found that corn starch in the diet inhibited the rise usually seen with simpler sugars. Certainly this might be a partial explanation for the low levels of cholesterol seen in Group I, but Group IV received an equal supplement of corn starch in the extracted germ without any impairment of the hypercholesteremic response. It seems most likely that an unidentified fraction of the germ oil, not easily extracted, is inhibiting the expected rise in serum cholesterol.

Summary. Dietary experiments in the cholesterol-fed chick have demonstrated that a higher level of serum cholesterol is achieved when cottonseed oil is fed instead of corn oil. Reconstituting the distilled fatty acids of cottonseed oil, and adding a small amount of myristic acid so that the proportions of the major fatty acids in corn oil were observed, did not make cottonseed oil fatty acids behave like corn oil in restricting the rise in serum cholesterol. Comparison of the whole corn germ with expressed and solvent extracted corn oil revealed that it was a much more potent agent for limiting the hypercholesteremia and subsequent atherosclerosis than the more refined products. It seems unlikely that this difference between corn oil and cottonseed oil in the effect on the hypercholesteremic response of the cholesterol fed bird could be explained by a difference in fatty acid composition.

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1. Ahrens, E. H., Jr., Blankenhorn, D. H., and Tsaltas, T. T., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 872.

2. Ahrens, E. H., Jr., Tsaltas, T. T., Hirsch, J., and Insull, W., *J. Clin. Invest.*, 1955, v34, 918.

3. Kinsell, L. W., Partridge, J., Boling, L., Margen, S., and Michaels, G., *J. Clin. Endocrinal. and Metabol.*, 1952, v12, 909.

4. Kinsell, L. W., Michaels, G., Partridge, J., Boling, L., Balich, H. E., and Cochrane, C. C., *J. Clin. Nutr.*, 1953, v1, 124.

5. Cochrane, G. C., Michaels, C., and Kinsell, L. W., *ibid.*, 1953, v1, 295.

6. Friskey, R. W., Michaels, G. D., and Kinsell, L. W., *Circulation*, 1955, v12, 492.

7. Bronte-Stewart, B., Antonio, A., Eales, L., and Brock, J. F., *Lancet*, 1956, v270, 521.

8. Jones, R. J., Kraft, S. C., Huffman, S., Balter, E. L., and Gordon, R., *Circulation Res.*, 1953, v1, 530.

9. Abell, L. L., Levy, B. B., Brodie, B. B., and Kendall, F. E., *J. Biol. Chem.*, 1952, v195, 357.

10. Horlick, L., and Katz, L. N., *Am. Heart J.*, 1949, v38, 25.

11. Stamler, J., Bolene, C., Levison, E., Dudley, M., and Katz, L. N., *Am. J. Physiol.*, 1948, v155, 470.

12. Swell, L., and Flick, D. F., *ibid.*, 1953, v174. 51.

13. Swell, L., Flick, D. F., Field, H., Jr., and Treadwell C. R., *ibid.*, 1955, v180, 124.

14. Lin, Tsung-Min, Karvinen, E., and Ivy, A. C., *ibid.*, 1955, v183, 86.

15. Portman, O. W., Laury, E. Y., and Bruno, D., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 321.

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Studies on Connective Tissue of the Cock Comb. II. Effect of Androgens.* (22673)

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Although the capon comb test has been used extensively for the biological assay of androgens, relatively little is known of the mechanism of this reaction in terms of tissue structure. The appearance of a so-called mucoid connective tissue has been recognized as the final result of hormone stimulation(1). An increase in the water content of the comb has been observed and is considered to be an important factor in the volume increase; based on this, the term "expansion growth" has been suggested for this reaction(2,3). Proliferation of the connective tissue cells does not seem to contribute significantly to the volume increase(3). Earlier histological studies indicated that mucopolysaccharides are present in the ground substance of the connective tissue of the rooster comb; this has been confirmed by the isolation of hyaluronic acid from the comb(4). It has been found that testosterone treatment increases the hexosamine content of the young chicken comb(5), but no similar studies have been reported on the capon comb after hormone treatment. Comparing the hexosamine content of the capon with that of the rooster comb, we found considerably higher values in the latter, while histochemical evidence indicated that acid mucopolysaccharides are present only in the rooster comb(6). The aim of the present study was to investigate the histochemical changes in the capon comb after testosterone treatment and to correlate these changes with the results obtained from determinations of the hexosamine, hydroxyproline and water content of the comb. The other head appendices, *i.e.* the wattles and the earlobes, have also been included in this study. Both are known to react to hormone treatment. However, while the comb and the wattles are similar in structure, the earlobes are made up of a morphologically different tissue. We also extended this investigation to the study of the normal development of the comb in untreated young cockerels.

Material and methods. 18 White Leghorn capons, approximately one year old were castrated at the age of 4 to 6 weeks and treated, with the exception of 4 control animals, with daily doses of 2 mg testosterone propionate (*"Neohombreol," Organon*)‡ for periods of 3 to 13 days. For comparison, the data obtained on 10 White Leghorn roosters of the same age are included. The development of the comb was studied in cockerels from the time they were hatched until 15 weeks of age, using a total of 82 animals.

Sampling the comb for a comparative study of its different stadia presents some difficulty due to the peculiar form of this organ and its anisometric volume increase. This is illustrated in Fig. 1, where the gradual increase in the area of the capon comb during hormone treatment is shown. It is obvious that in the course of this process each point of the comb moves at a different speed and in a different direction. By taking transverse slices from the comb at the plane of arrow A (Fig. 1), one can obtain sections which represent analogous parts of the comb in different animals. In studying such sections of the capon comb after different periods of hormone treatment, we found that the changes in the connective tissue start at the base of the comb and proceed upward from the skull toward the spikes(3). Such transverse sections might have obvious advantages for a morphological and a histochemical study. However, because the composition of these sections is not uniform, correlation of the results of chemical analysis

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* The first article of this series appeared in *J. Histochem. and Cytochem.*, 1956, v4, 97.

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‡ Generously supplied by N. V. Organon, Oss, Netherlands.

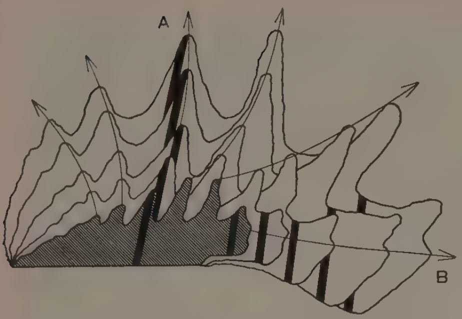


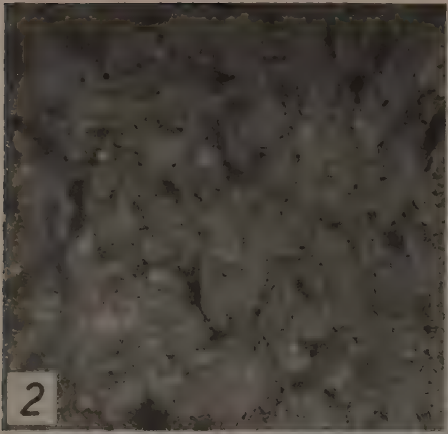
FIG. 1. Schematic representation of increase of comb area in the capon in the course of testosterone treatment (2 mg daily). Based on contact photographs taken every second day during first 8 days of treatment. Heavy black lines indicate location of samples.

with the histological structure is much more difficult. For this latter purpose, we used comb slices cut perpendicularly to arrow B (Fig. 1), which represent a more homogeneous cross section of the process at different time intervals.

The histological and histochemical methods, including the sectioning of the fresh frozen tissue, the periodic acid-Schiff reaction (PAS) and metachromatic staining with toluidine blue, have been described in detail(6). In a few cases the tissue, after fixation in Pfuhl's mixture (sublimate, picric acid, glacial acetic acid), was embedded in paraffin and stained with haematoxylin-phloxin or azocarmin-orange G-aniline blue (AZAN). Hexosamine determinations were carried out according to the modified Elson-Morgan method described previously(6). Comb slices of about 300 mg were used for this purpose except in very young cockerels, where it was necessary to use the whole comb or even pooled samples of several combs. Prior to hydrolysis, the tissue samples were extracted by acetone and dried in a desiccator to constant weight. This dry weight of the defatted tissue has been used to calculate the water content. The method described by Neuman and Logan(7) has been used for the determination of the hydroxyproline content. The data reported on the hexosamine, hydroxyproline and water content represent averages of determinations made on 10 combs of roosters, 4 combs of untreated capons, and 2 combs from each group of treated capons; while in the case of cockerels, the number of combs used varied between 3 and 8. The ratio of hexosamine to hydroxyproline has also been calculated.

Results. Testosterone-treated capons. The average weight of the untreated capon comb was 1.57 g, while the rooster combs measured about 45 g. After 13 days of testosterone treatment of capons, the average comb weight was 21.2 g. A detailed description of the connective tissue of untreated capon comb is given in previous reports(3,6); here we shall mention only that there is no metachromatic ground substance present in the capon comb and that the hexosamine content is rather low (0.53% dry weight). Estimating the hydroxyproline content in the comb of untreated capons, we found an average value of 3.65% dry weight, resulting in a ratio hexosamine: hydroxyproline of 0.15. Studying the changes in the capon comb after testosterone treatment, we found after 3 days that there was already a large amount of metachromatic material present in the intermediate layer, especially in the lower parts of the comb. The gradual development of this mucoid tissue is shown in Fig. 2, 3 and 4, which are taken from different parts of the same transverse section through the middle of the comb. Fig. 2 shows the very first appearance of the metachromasia in the spike; Fig. 3 shows further development in the middle part; while Fig. 4 represents a fully developed mucoid tissue at the base of the comb close to the skull. In comparing sections from the combs of capons after different periods of treatment, 3, 5 and 13 days respectively, we found that after 3 days the fully developed mucoid tissue is restricted to the basal parts, while after 5 days almost the whole section contains this layer. Finally, after 13 days, the intermediate layer throughout the entire comb is transformed into mucoid tissue, occupying all of the space between the reticular layer under the epithelium and the central core. The appearance of the metachromasia is the main occurrence histochemically. We did not find any change in the PAS positivity, which was restricted to the collagen fibers(6).

The wattles show essentially the same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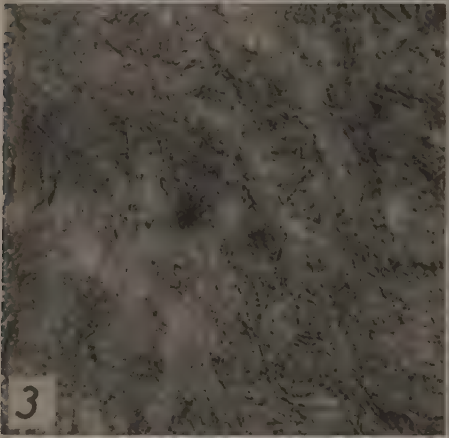
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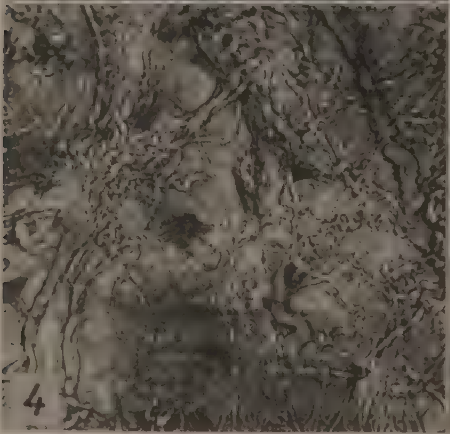
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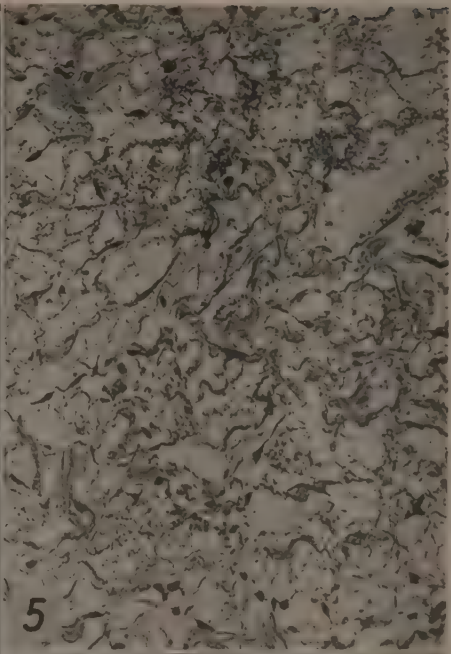
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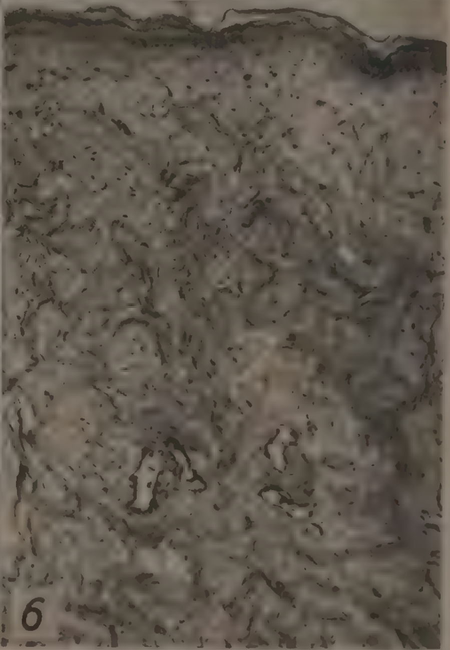
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FIG. 2. Capon comb after 3 days of hormone treatment. Frozen section 12.5 μ, fixation in osmic vapor and lead subacetate, stained with toluidine blue (magnification 360 ×). Mucoid layer in the spike.

FIG. 3. Same section and magnification as in Fig. 2. Mucoid layer in middle part of comb.

FIG. 4. Same section and magnification as in Fig. 2. Mucoid layer in basal part of comb near skull.

FIG. 5. Subcutaneous connective tissue of the wattle of a capon after 3 days of testosterone treatment, showing edema. Fixation: Pfuhl, paraffin section 8 μ, stained with AZAN (magnification 320×).

FIG. 6. Earlobe of untreated one-year-old rooster. Fixation: Pfuhl, paraffin section 6 μ, stained with haematoxylin-phloxin (magnification 180×). Note absence of blood vessels under epithelium and presence of thick, collagen bundles in dermal layer.

structure as the comb, both in the capon and in the rooster, and the reaction to testosterone treatment is very similar. It should be mentioned that the mucoid layer here is narrower than in the comb. Therefore, the mucoid tissue represents a smaller portion of the total structure. There is no preferential localization of the very first metachromatic change in the wattles, as in the basal part of the comb. We noticed another way in which the reaction here seems to differ from that in the comb; *viz.* the edematous appearance of the subcutaneous tissue of the wattle, which was especially pronounced during the first days of hormone treatment. At this time, the wattle increases markedly in thickness, and on cutting through the tissue, fluid escapes from the central part. This fluid showed strong metachromasia when toluidine blue was added *in vitro*, but it was technically impossible to demonstrate this substance in the tissue sections. The spaces between the fibrils of this edematous middle part appear empty in the histological preparation (Fig. 5). Later on this edema tends to disappear; after 13 days of treatment, the subcutaneous tissue of the wattles appears to be similar to the structure in the rooster without any sign of edema.

Histologically, the earlobes of both the capon and the rooster differ from the comb and wattle in many ways. In the subepithelial part of the rooster comb and wattle, the so-called reticular layer, there is a large number of sinusoid blood vessels, showing dilatation upon hormone treatment and giving these organs their characteristic red color. These subepithelial vessels are absent in the earlobes, where there is only a small strip of tissue somewhat similar to the reticular layer of the comb and wattle. While the dermal layer of the comb and wattle of the normal rooster and treated capon contains thin collagen fibrils, the dermal layer of the earlobes consists of thick collagen bundles, homogeneous in appearance. A few small, coiled blood vessels are also present here. (Fig. 6). Compared to the very profound reversible changes of the dermal layer of the comb under hormone treatment, the dermal layer of the earlobes shows fewer alterations. In the capon, the whole dermal layer is narrower and there is no metachromasia present. In the testosterone-treated capon and in the normal rooster, this layer is wider, the collagen bundles have a swollen appearance, and diffuse metachromasia, very faint but still distinct, is present throughout the whole dermis. The only effect of hormone treatment on the earlobe which is similar to that on the wattle is some accumulation of fluid in the subcutaneous tissue, but to a much lesser extent.

The results of chemical analysis of all 3 tissues in the treated capons are summarized in Fig. 7. It is obvious that there is a significant increase in the total hexosamine content of the capon comb during hormone treatment. The same is true for the wattles, but to a lesser degree, while in the earlobes the increase in hexosamine, if at all significant, is very slight. The relative amount of hydroxyproline does not change significantly in the capon comb during hormone treatment; this also seems to apply to the wattles and earlobes. When these values are related to each other, as the hexosamine:hydroxyproline ratio, it is obvious that this ratio tends to increase in all 3 organs during testosterone treatment; these values can be even higher than in the normal rooster.

Normal development of the comb in cockerels. In our experiments, the comb weight increased from 0.013 g at the time of hatching to about 15 g at the age of 15 weeks. The histological structure of the comb in a one-day-old cockerel shows a cellular proliferation of the connective tissue of the dermal layer;

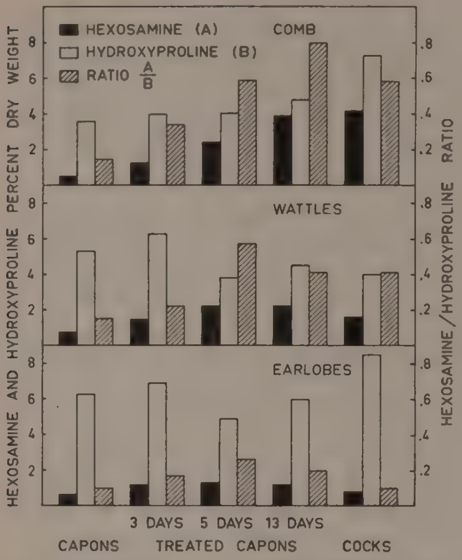

FIG. 7. Hexosamine and hydroxyproline content and hexosamine-hydroxyproline ratio in comb, wattles and earlobes of testosterone-treated capons. For comparison, values from untreated capons and roosters of the same age are included.

the collagen fibrils are already developed, but there is no metachromasia in the ground substance. This picture does not change essentially during the first 3-4 weeks, which seems to cover the normal growth period judging from the frequent mitoses observed both in epithelium and in connective tissue. After this period the accumulation of the metachromatic ground substance begins and is again more pronounced in the basal parts of the comb. Until the age of 15 weeks there is a gradual increase in the amount of mucoid tissue in the comb. During this time mitoses still can be seen in the epithelium, but are seldom observed in the connective tissue. At the end of this period the structure of the normal rooster comb has been developed. Chemical determinations (Fig. 8) show that the relative amount of hexosamine in the comb starts to increase very slowly during the first 4 weeks, but increases more rapidly thereafter, reaching the level of the fullgrown rooster comb in about 15 weeks. The relative amount of hydroxyproline is constant throughout the whole development. The hexosamine-hydroxyproline ratio conceivably

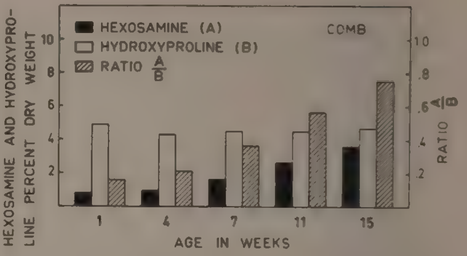

FIG. 8. Hexosamine and hydroxyproline content and hexosamine-hydroxyproline ratio in comb of developing cockerels.

increases during this time.

In Fig. 9 water content data of comb samples are plotted against the corresponding hexosamine values. These figures show that in treated capons the water content is somewhat higher than in normal, untreated roosters and older cockerels.

Discussion. The comparative histological study of the comb development in cockerels and in testosterone-treated capons showed that in both cases the process of "expansion growth" is essentially similar. This process takes 10 to 15 weeks in cockerels; in treated capons it can be completed in about 2 weeks.

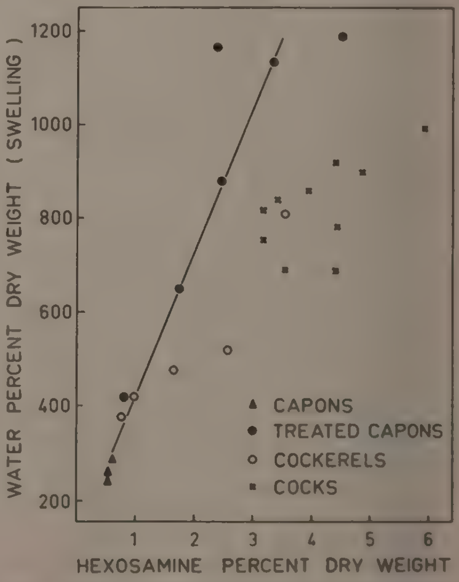

FIG. 9. Water content (expressed in % of fat-free dry wt) and hexosamine content of comb in treated and untreated animals.

In the cockerel, it is preceded by a phase of normal tissue growth which takes place during the first 3-4 weeks after hatching. Mitoses in the connective tissue were found only during the first 4 weeks; after that, during the "expansion growth" period, we rarely found cell division in the mucoid layer. Ludwig and Boas(8) and Schiller, Benditt and Dorfman (5) observed active cell proliferation in the connective tissue of the comb in young cockerels receiving androgen treatment. The animals used were 3-24 days old(8) and 10 days old(5) respectively. A possible explanation for this difference could be that in animals so young the process of "expansion growth" overlaps the normal growth process. Determination of the total hexosamine shows that there is an increase of some hexosamine-containing substance or substances in both treated capons and normally developing cockerels in the course of the "expansion growth." This applies especially to the comb, but also seems to occur in the wattles and possibly in the earlobes. It should be mentioned that our data do not allow any further interpretation of this increase in hexosamine content, but they are in all cases parallel to the metachromasia as found under the conditions of the histochemical test. This altogether might be evaluated as indirect evidence for the supposition that one of the main phenomena in the hormonal response of these tissues is the accumulation of mucopolysaccharides in the ground substance, probably hyaluronic acid. Since the concentration of hydroxyproline in the treated capon remains relatively constant in the course of the "expansion growth," this indicates that there is also an active formation of collagen during the process which is proportional to the increase of the total fat-free dry mass of the comb. Compared to this the increase in hexosamine content is much larger. The same applies to the increase in water content, which is parallel to the increase in hexosamine content, suggesting that these two phenomena are in some way related to each other(2). This is supported indirectly by the previous observation that the water content of the isolated mucoid layer of the rooster comb as well as its hexosamine content are much higher than the values obtained on the total comb(6). Another factor which might partially explain the high water content of these organs is the subcutaneous edema which develops in the course of hormone treatment, especially in the wattles and earlobes. Finally, the vasodilatation will increase the blood content of the tissue and also may contribute to the high water content.

Summary. It has been found that the increase of the metachromasia in the connective tissue of the comb coincides with the increase of the total hexosamine content during the development of this organ in the normal rooster, as well as during the testosterone treatment of the capon. In both cases, there is an increase in the water content, suggesting that the presence of mucopolysaccharides in this tissue and the high water content are related phenomena. The relative hydroxyproline values remain essentially unchanged during this reaction, indicating that increase in collagen content is proportional to total increase of the dry substance. The ratio hexosamine:hydroxyproline thus shows an increase in both cases. A similar increase was also found in the wattles and, to a lesser degree, in the earlobes. The wattles show the same structure and reaction as the comb. In the earlobes there is a very slight increase of the metachromasia in the course of hormone treatment which, together with the slight increase in the hexosamine:hydroxyproline ratio, would suggest that the reaction here is also similar, but much less pronounced. During testosterone treatment a transient edema of the subcutaneous tissue has been found, especially in the wattles and earlobes.

The author wishes to thank Dr. G. Smits of the Histological Laboratory, Municipal University of Amsterdam, Netherlands, for his cooperation and advice in the chemical part of this work.

1. Champy, Ch., *C. R. Soc. Biol.*, 1926, v94, 311.

2. Heringa, G. C., *Proc. 6th Internat. Cong. Exp. Cytol. (Stockholm)*, 1947, 366.

3. Szirmai, J. A., *Anat. Rec.*, 1949, v105, 337.

4. Boas, N. F., *J. Biol. Chem.*, 1949, v181, 573.

5. Schiller, S., Benditt, E. P., and Dorfman, A., *Endocrinology*, 1952, v50, 504.

6. Szirmai, J. A., *J. Histochem. and Cytochem.*, 1956, v4, 97.

7. Neuman, R. E., and Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.

8. Ludwig, A. W., and Boas, N. F., *Endocrinology*, 1950, v46, 291.

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Effect of X-Ray Dose on Mortality and Skin Transplantability in Mice Receiving F_1 Hybrid Marrow.* (22674)

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Mice receiving an otherwise lethal dose of X-irradiation, but protected with bone marrow from an F_1 hybrid derived from the irradiated and a foreign strain, will subsequently tolerate skin homografts from the F_1 hybrid or the foreign parent strain of the F_1 hybrid(1,2). Mice protected with marrow from the same (isologous) strain will not tolerate skin homografts from the F_1 hybrid or the foreign parent strain. The following experiments were undertaken to determine the lowest dose of whole body X-irradiation, followed by marrow from an F_1 hybrid, that would result in tolerance of skin homografts from the foreign parent strain. During the course of these experiments an entirely unexpected and interesting observation was made regarding mortality in marrow-treated mice at decreasing doses of irradiation.

Materials, methods and terminology are similar to those reported previously(2) with the following exceptions. One marrow donor

TABLE I. Percent Mortality following X-irradiation.

	All strains combined			CBA strain		
		% mortality after indicated			% mortality after indicated	
Dose, r	No. of mice	No. of days 21	30	No. of mice	No. of days 21	30
330	22	0	0	12	0	0
400	12	0	0			
500	24	17	21			
550	54	9	9	24	12	12
600	36	64	67	12	50	50
660	84	93	94	48	90	90
700	24	100	100			
770	266*	99	100	60	100	100
800	50	100	100	11	100	100
1000	12	100	100			
1200	12	100	100			

* 159 of the mice at 770 r received intrav. saline inj. as controls for other groups receiving bone marrow suspensions.

TABLE II. Mortality and Skin Transplantability in Unirradiated, X-irradiated and Marrow Treated CBA Mice.

									Skin graft				
Exp.	X-ray dose, r	Marrow donor	No. of mice	% mortality after indicated No. of days 12	21	30	60	100	Skin donor strain	No. of mice grafted	Days post-X-ray and/or marrow when grafted	No. of takes	No. of sloughs
A	0	Cb × CBA	12	0	0	0	0		Cb	12	46–52	0	12
	110	*Idem*	12	0	0	0	0		”	12	46–52	0	12
	330	”	12	0	0	0	0		”	12	46–52	0	12
	550	”	12	67	100	100	100	100		0			
	770	”	12	0	0	0	8*		Cb	11*	46–52	11	0
	0	None							CBA	13		12	1
B	660	Cb × CBA	12	0	0	17	42†	50	Cb	9	34–55	6†	0
	770	*Idem*	12	0	0	0	0	0	”	12	34–55	9	3
	660	None	12	67	100	100	100	100		0			
	0	”							Cb	9		0	9
	0	”							CBA	21		21	0

* One mouse in this group died the day following skin grafting and is excluded from the skin graft data but included in the mortality data.

† Three mice in this group died (one as a result of anesthesia during photography) with viable skin grafts 13 to 18 days after skin grafting. They are excluded from the skin graft data but included in the mortality data.

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† The author is indebted to Dr. R. Shalek for assistance in dosimetry, and to M. A. Flores and B. Morris for technical assistance. X-ray facilities were generously provided by the M. D. Anderson Hospital and Tumor Institute.

TABLE III. Mortality and Blood Count in X-irradiated CBA Mice Receiving Cb × CBA F₁ Marrow.

Strain of mice	X-ray dose, r	Marrow donor	No. of mice	% mortality after indicated No. of days 12	21	30	Blood counts at 2 wk post-irradiation: No. of mice counted	WBC/mm³ Mean	WBC/mm³ Low	WBC/mm³ High	Million RBC/mm³ Mean	Million RBC/mm³ Low	Million RBC/mm³ High
CBA	330	Cb × CBA	12	0	0	0							
"	550	*Idem*	12	33	92	92	5	3,430	800	7,400	7.33	3.18	11.75
"	770	"	12	8	8	17	5	9,190	8,500	11,950	11.49	8.65	13.63
Balb/c*	770	None	12	100	100	100							

* Balb/c mice were used as an irradiation control because of a shortage of CBA mice.

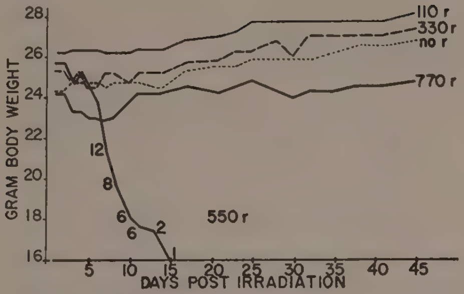

FIG. 1. Body wt data of Exp. A, Table II. All points represent mean wt of 12 mice with the exception of the 550 r group. Numbers along this line represent decreasing numbers of surviving mice at each point.

mouse was used for every 2 irradiated mice in Experiment A of Table II and in the experiment of Table III, rather than 1 for 1 as in previous work, including Experiment B of Table II. The intravenous injection volume was .5 cc throughout. The nucleated cell counts for the marrow suspensions used were 67,200, 89,150 and 61,250 per cu mm for Experiments A and B of Table II and for Table III respectively. Duco cement was applied to the sutured edges of the skin grafts of Experiment A, but not of Experiment B.

Observations. The 21-day LD_{50} of X-irradiation under the conditions used is about 600 r, while 770 r is uniformly lethal to all mice within 30 days (Table I) Mice of the CBA strain were equally distributed by age and sex into 5 treatment groups, each containing 12 mice. These groups received 0, 110, 330, 550, and 770 r of X-irradiation followed in 1 to 2 hours by intravenous injection of marrow from the Cb x CBA F_1 hybrid. As in past experience(2), F_1 marrow gave excellent protection against the otherwise lethal dose of 770 r (Experiment A of Table II). At the lower dose of 550 r, however, administration of the same suspension of hybrid marrow was followed by 100% mortality in 15 days. Body weight loss at 550 r was much more severe than at 770 r and was progressive until death (Fig. 1). Mice receiving still lower doses of irradiation or no irradiation, followed by marrow treatment, showed no mortality. At 46 to 52 days post-irradiation the surviving mice were homografted with skin from the Cb strain, the other parent strain of the F_1 marrow donor. Of a group of 13 untreated CBA mice simultaneously autografted as controls, 12 of the autografts took. All of the homografts sloughed at the 0, 110 and 330 r dose levels. Of the 11 mice at 770 r that survived skin grafting, all of the foreign skin grafts were accepted and are in good condition 124 to 130 days after grafting. In previous experiments no late sloughs have been observed under similar conditions. Fifteen of 18 grafts at 660 and 770 r were successful and are still in good condition 130 to 152 days after grafting (Experiment B of Table II). In this earlier experiment it will be noted that late mortality in F_1 marrow protected mice at 660 r was greater than at 770 r. In both of these experiments mice receiving the different doses of irradiation were caged separately, 6 to a pen. It is therefore possible that an undetected infectious agent may have involved the 4 pens of mice at 550 and 660 r to account for the greater mortality at these levels than at 770 r. To control this possibility another experiment was set up in which mice receiving 330, 550 and 770 r followed by Cb x CBA marrow were caged together, 2 at each dose level in each pen of 6 mice. Again the mice at 550 r showed much higher mortality than their cage mates at 330 and 770 r (Table III). Body weight loss at 550 r was much greater and more progressive than at 770 r. Blood counts performed 2 weeks post-irradiation showed a significantly lower white and red cell count in the mice at 550 r than in those at 770 r. Platelets were less numerous in the stained blood smears of the mice at 550 r than in those at 770 r.

Discussion. The blood count data suggest that the greater mortality at 550 r than at 770 r may be related to a less adequate hematopoietic recovery. On the other hand, 550 r without bone marrow had earlier resulted in 30-day mortality of only 12% of 24 CBA mice and 9% of 54 mice of all strains combined. Additional experiments are obviously indicated, and no explanation of the mechanism involved will be ventured at this time.

Summary. The 21-day LD_{50} under the conditions of X-irradiation used is approximately 600 r. Irradiation of CBA mice followed by intravenous administration of bone marrow suspension from Cb x CBA F_1 hybrids resulted in little or no 21-day mortality at 110, 330, 660 and 770 r, but in almost 100% mortality at 550 r. Homografts of Cb skin into the surviving mice were accepted by the mice at 660 and 770 r dose levels but not by mice at 0, 110 and 330 r dose levels. None of the marrow-treated mice at 550 r survived long enough to be skin grafted.

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1. Main, J. M., and Prehn, R. T., *J. Nat. Cancer Inst.*, 1955, v15, 1023.

2. Trentin, J. J., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 688.

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Effect of Chlorpromazine on Excretion of 5-Hydroxyindoleacetic Acid in a Patient with Malignant Carcinoid. (22675)

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Since the first authentic case report of Beger(1) in 1882 of a carcinoid of the appendix the tumor has been of interest to the clinician principally because of its rarity and to the histologist because of its unusual cell type and staining properties. Gosset and Masson(2), using silver impregnation techniques, demonstrated the presence of intracytoplasmic granules which stain black with silver and which were indistinguishable from the granules found in the argentaffin cells of the intestinal mucosa. Masson suggested the possibility that carcinoid tumors arise from the argentaffin cells and postulated that the normal cells had a neuroendocrine function.

Lembeck(3) demonstrated the presence of 5-hydroxytryptamine (5-HT) in carcinoid tumors in large quantities. This observation has led Thorson(4) to suggest that the secretion of 5-hydroxytryptamine (serotonin) by the tumor might serve to explain the unusual symptom complex seen in patients harboring this tumor. The clinical syndrome is characterized by intestinal hypermotility, bronchospasm, vasomotor disturbances and valvular disease of the heart. The recent report of Sjoerdsma(5) *et al.* concerning their investigation on a group of patients with malignant carcinoids support the postulate that the symptom complex described by Thorson and others is due to excess serotonin produced by the tumor. Further studies by the group have shown that 5-hydroxyindoleacetic acid (5-HIAA) is formed by oxidative deamination of serotonin and excreted in the urine(6). Increased amounts of this metabolite have been demonstrated in the urine of patients with malignant carcinoid and a method for its assay has been described by Udenfriend(7). The recent reports of Benditt(8) showing antagonism of 5-HT by chlorpromazine led us to study the effects of this drug on a patient with a known malignant carcinoid.

Method. The patient was a 56-year-old white woman in whom a carcinoid tumor of the ileum was diagnosed and resected in 1950. At the time of laparotomy metastases of the tumor to regional lymph nodes and liver were observed grossly and confirmed microscopically. She has enjoyed reasonably good health since that time with the exception of chronic diarrhea, occasional episodes of "flushing" and periodic exacerbations of "arthritis." No cardiac abnormalities have been noted. The patient was admitted to the Metabolic Division of University Hospitals and placed on a constant diet which remained unchanged during the study period. All urine was collected and aliquots from each 24-hour period were placed in a deep freeze. The number and character of the bowel movements were recorded for each 24 hours. Following a control period of 5½ days the patient was started on oral chlorpromazine,* 25 mg every 6 hours. This was continued for 4 days at which time the drug was discontinued. The observation period extended for an additional 4½ days following withdrawal of the medication. The 24-hour urine samples were assayed for 5-HIAA according to the simplified method of Sjoerdsma *et al.*(9). A gross quantitation of the amount of 5-HIAA excreted was made by the intensity of the color produced by the test and was adequate for our purpose. The patient received no other medication during the experimental period.

Results. Following administration of chlorpromazine there was a prompt and marked decrease in the excretion of 5-HIAA in the urine and a decrease in the number of bowel movements per 24 hours (Fig. 1). The stools became semi-solid in contrast to watery bowel movements observed during the control period. Within 24 hours following discontinuation of the medication the urine 5-HIAA content returned to control levels and the diarrhea again became manifest after 72 hours.

Discussion. The evidence that carcinoid tumors produce excess quantities of serotonin seems incontrovertible and patients with this lesion afford an unusual opportunity to study the pharmacodynamic effects of this substance *in vivo*. The patient described in this study may be added to a growing list of reported cases in whom the presence of this tumor is associated with unusual clinical manifestations which may be attributable to serotonin excess, *i.e.*, diarrhea, vasomotor disturbances (flushing), etc. The antagonistic effect of chlorpromazine on serotonin previously demonstrated in experimental animals(8) is further supported by this study as shown by the decrease in urinary 5-HIAA following administration of the drug. The decrease in intestinal hypermotility as manifested by the reduction in the number of stools noted in this patient affords additional corroborative evidence of serotonin inhibition. The mode of action of serotonin inhibition by chlorpromazine remains speculative. It is not to be inferred from this report that chlorpromazine is necessarily recommended in the clinical management of these patients albeit true that symptomatic relief of the distressing diarrhea

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* Thorazine—Smith, Kline and French.

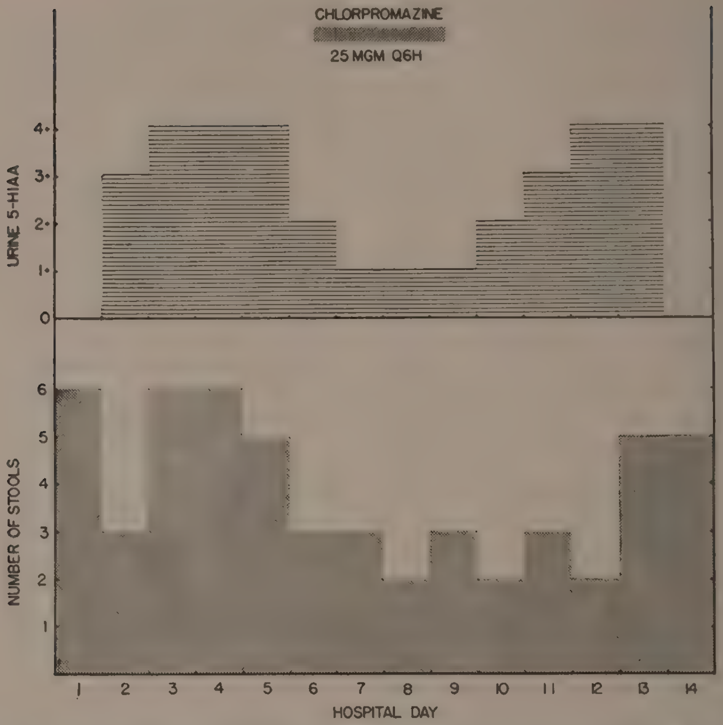

FIG. 1. Graph showing prompt fall in the urine 5-hydroxyindoleacetic acid following oral administration of chlorpromazine and a return to premedication levels following withdrawal of the medication. Concomitant diminution in the No. of stools is also depicted occurring with chlorpromazine medication.

was observed.

Summary. 1. A marked fall in urinary 5-HIAA excretion followed administration of chlorpromazine to a patient with known malignant carcinoid. 2. Intestinal hypermotility, one manifestation of the so-called "carcinoid syndrome" was likewise suppressed during administration of the drug.

The authors are grateful for the helpful suggestions of Dr. Jack Leonards, D. Stanley Levey, and Dr. Albert Sjoerdsma.

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1. Beger, A., *Klin. Wchnschr.*, 1882, v19, 616.

2. Gosset, A., and Masson, P., *Presse med.*, 1914, v25, 237.

3. Lembeck, F., *Nature, London,* 1953, v172, 910.

4. Thorson, A., Biorck, G., Bjorkman, C., and Waldenstrom, J., *Am. Heart J.*, 1954, v47, 795.

5. Sjoerdsma, A., Weissbach, H., and Udenfriend, S., *Am. J. Med.*, 1956, v20, 520.

6. Sjoerdsma, A., Smith, T. E., Stevenson, T. D., and Udenfriend, S., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 36.

7. Udenfriend, S., Titus, E., and Weissbach, H., *J Biol. Chem.*, 1955, v216, 499.

8. Benditt, E. P., and Rowley, D. A., *Science*, 1956, v23, 24.

9. Sjoerdsma, A., Weissbach, H., and Udenfriend, S., *J.A.M.A.*, 1955, v159, 397.

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Stability of a Fluid Cholera Mucinase Preparation When Combined with a Commercially Prepared Cholera Vaccine. (22676)

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The demonstration by Burnet and Stone (1) that the mucinase of *Vibrio comma* causes desquamation of guinea pig intestinal epithelium suggested to these investigators that this enzyme may be involved in the pathogenesis of Asiatic cholera. Evidence to support this hypothesis is provided by the recent report of Lam, Mandle and Goodner(2) that the cholera mucinase increases the permeability of the mouse intestinal wall *in vivo* as well as *in vitro*. It is postulated that the mucinase, by causing desquamation of the intestinal epithelium, alters the permeability of the bowel wall to permit absorption of toxic substances and loss of fluids and electrolytes.

The usual cholera vaccine, which consists of non-viable vibrio cells, does not stimulate production of antimucinase when injected into animals(1,3). Vibrio culture filtrates containing active mucinase, on the other hand, are antigenic when administered parenterally to rabbits and guinea pigs, and result in the appearance of specific antibodies which inhibit the *in vitro* desquamation and depolymerization activities of the homologous mucinolytic enzyme(1,4). It would appear, then, that the addition of cholera mucinase to the vaccine would enhance the protective potency of this immunizing agent against an enteric cholera infection, provided the mucinase retains its antigenic properties in such a preparation.

Jensen(3) studied the stability of the cholera mucinase in culture filtrates and reported that the enzyme in this state is extremely sensitive to heat, storage and chemical preservatives. He demonstrated that *in vitro* enzyme activity and antigenicity are related, and that both properties can be preserved by lyophilization.

The studies reported herein were initiated to ascertain whether the application of a concentration and purification procedure would result in a stabilized fluid mucinase preparation, permitting the combination of the mucinase with the present cholera vaccine without affecting the *in vitro* activity and antigenicity of the enzyme, or the antigenicity of the somatic antigens.

Materials and methods. Culture: *V. comma* strain 20A78, the WRAIR designation for strain P93A obtained from Prof. K. Goodner, Jefferson Medical College, Philadelphia, Pa., was used for the preparation of mucinase by the method described below. *Cholera vaccine*: Lederle Cholera Vaccine, Lot #1976-67K (expiration date 21 Oct. 1955), was obtained in Nov. 1954 for use in these studies. The vaccine contained 8,000 million killed cholera vibrios (equal numbers of Inaba and Ogawa strains) per ml, preserved in 0.45% phenol. *Mucinase titrations*: *In vitro* mucinolytic activity was measured by the technic described elsewhere(5), which is based on the ability of the mucinase to depolymerize ovomucin so that the latter is no longer clotted by cetyl trimethyl ammonium bromide (CTAB). The ovomucin was prepared by the method of Gottschalk and Lind(6) and was standardized by the addition of borate-buffered calcium saline, pH 7.0,* to the maximum dilution containing sufficient dissolved ovomucin to form a characteristic fibrous clot on addition of one drop of 1% aqueous CTAB to 0.5 ml of the standard solution in 0.5 ml of buffered saline. The mucinase titers recorded are the reciprocals of the highest dilutions of the enzyme solutions which prevented the formation of the ovomucin-CTAB clot. *Antimucinase titrations*: The antimucinase activity of serum was measured by the constant antigen-varying antibody technic previously described (5). The titers recorded are the reciprocals of the highest serum dilutions which neutralized the depolymerizing activity of 2 mucinase units, permitting the formation of the ovomucin-CTAB clot. *Agglutinin titrations*: Serum agglutinin titers were obtained by employing as antigen a saline suspension of agar-grown viable vibrios of the same strain from which the mucinase was prepared (20A78). Antigen-antiserum mixtures were incubated at 37°C for 2 hours followed by refrigeration overnight. The titers recorded are the reciprocals of the highest dilutions of the sera which yielded definite macroscopic agglutination.

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* Borate-buffered calcium saline, pH 7.0: Per liter: 0.052 g $Na_2B_4O_7 \cdot 10H_2O$, 1.0 g $CaCl_2$ (anhydrous), 8.5 g NaCl, 1.203 g H_3BO_3.

Experimental results. Purification of Mucinase: In order to facilitate purification of the mucinase from culture filtrates an attempt was made to cultivate the cholera vibrio in a protein-free medium. Several synthetic media and media containing protein hydrolysates were tried, and although good yields of bacterial cells were obtained in many instances, little or no mucinolytic activity was demonstrated in the culture supernates. On the other hand, a dialysate of Brain-Heart Infusion Broth was found to support the growth of the organism and to yield satisfactory mucinase titers, and was consequently used as the culture medium.

Double strength Brain-Heart Infusion Broth (Difco) was dialyzed against an equal volume of distilled water at 4°C. After 24 hours the dialysate was collected and was replaced with another volume of water. This process was repeated again, after which the 3 resulting dialysate solutions were combined to constitute the medium for the growth of the vibrios. Nine liter bottles containing 2 liters of dialysate medium were seeded with 50 ml of a 6 hour vibrio culture (in the same medium) and were incubated at 37°C for 17 hours on a roller apparatus which constantly rotated the bottles (tilted at an angle of 30 degrees) at 40 revolutions per minute throughout the incubation period, providing maximum aeration without excessive foaming.

The bulk of the bacterial cells was removed by centrifugation and the remainder by filtration through a large Berkefeld filter of N (normal) porosity. The filtrate was then half-saturated with solid $(NH_4)_2SO_4$ and was placed in the cold room overnight. The resulting sediment was dissolved in borate-buffered calcium saline and was dialyzed against the buffered saline until the dialysate no longer precipitated with a saturated barium chloride solution. Sterilization of the product was accomplished by filtration through a Selas filter (02 porosity).

The final solution contained mucinase, other non-dialyzable ammonium sulfate precipitated extracellular products of vibrio metabolism, and cellular substances (somatic antigens, etc.) liberated through lysis of the cells. The material used in this study was prepared in May 1953 from culture filtrates of *V. comma* strain 20A78. The original culture filtrate contained 1.48 mg of nitrogen per ml and had a mucinolytic activity of 64 units per ml, while the final product contained 0.14 mg of nitrogen and 2048 mucinase units per ml (Table I). Comparable results have since been obtained with other preparations from the same strain, as well as with preparations from 4 other *V. comma* strains, including a recent isolate from a human cholera case.

Stability of in vitro enzyme activity: The partially purified mucinase in borate buffered calcium saline, prepared as described above, proved to be extremely stable when stored in the fluid state at 4°C, retaining its full *in vitro* enzymatic activity for at least 2½ years (column I, Table II). In Nov. 1954, after 1½ years storage, this mucinase solution was mixed with stock commercially prepared cholera vaccine in 4 different proportions. These mixtures, in rubber stoppered vaccine bottles, were stored at 4°C for an additional year, with periodic assays for *in vitro* mucinase activity. No significant variation in titer, be-

TABLE I. Comparison of Vibrio Culture Filtrate and Partially Purified Mucinase Solution.

Preparation	Vol, ml	Mucinase titer/ml	Mg N/ml	Mucinase units/mg N	Yield
Culture filtrate	7800	64	1.48	43.2	
Purified mucinase	110	2048	.14	14629	45%

TABLE II. Mucinase Titers of Mucinase-Vaccine Mixtures.

	Mixture* No. 1	2	3	4	5
Mucinase solut'n†	20 ml	15 ml	10 ml	5 ml	1 ml
Cholera vaccine‡	0	5 "	10 "	15 "	19 "
Date tested			Mucinase titers		
5/18/53	2048	—	—	—	—
12/ 1/54	2048	1024	512	256	128
1/ 3/55	2048	1024	512	512	128
2/ 7/55	2048	2048	1024	512	128
3/21/55	2048	1024	1024	512	64
5/27/55	4096	2048	2048	1024	256
7/ 1/55	2048	1024	512	256	64
8/ 1/55	2048	1024	1024	512	64
9/30/55	2048	1024	1024	512	128
12/ 5/55	4096	2048	2048	1024	128

* Mixtures 2 through 5 prepared 11/24/54.

† Mucinase solution prepared in May 1953 from culture filtrate of *V. comma* strain 20A78.

‡ Lederle Cholera Vaccine, Lot No. 1976-67K. Mucinase titer = <4.

yond that inherent in a technic involving 2-fold serial dilutions of the enzyme solution and different preparations of the substrate, was noted with any of the mixtures during this period (Table II). *Stability of antigenic properties of the mucinase and of the somatic antigens*: Subsequent to storage at 4°C for one year, 3.25 ml of the mixture containing mucinase and vaccine in equal volumes was injected intravenously into 3 rabbits in 8 graded doses (ranging from 0.05 ml to 2.0 ml) at 3-4 day intervals. Simultaneously a second group of 3 rabbits received intravenous injections of 1.625 ml of the mucinase preparation alone and a third group was given 1.625 ml of the cholera vaccine alone, in graded doses ranging from 0.025 ml to 1.0 ml. The rabbits were bled 10 days following the final injection and the sera were assayed for agglutinin and antimucinase activity.

The antimucinase titers of the sera of rabbits immunized with the mucinase-vaccine mixture fell in the same range as those resulting from immunization with the mucinase solution alone (Table III). Thus there appears to be no decrease in the antigenic stability of mucinase as a result of its mixture with cholera vaccine, even after such a mixture has been stored for a year at 4°C. On the other hand, the appearance of agglutinins in the sera of these rabbits provides evidence that the presence of active mucinase in the vaccine does not destroy the antibody-stimulating properties of the vibrio somatic antigens.

The presence of agglutinins in the sera of the rabbits immunized with the mucinase solution alone is undoubtedly due to the precipitation of somatic antigens from the culture filtrate along with the mucinolytic enzyme during the preparation of the solution. Absorption of these sera with washed vibrios reduces the agglutinin titer without affecting the antimucinase activity(7). This, together with the observation that the cholera vaccine (containing non-viable cells) fails to stimulate the production of antimucinase, provides evidence that the mucinase and the somatic antigens of *V. comma* are not immunologically related, thus confirming the findings of Burnet and Stone(1) and Jensen(3).

Discussion. The role of the cholera mucinase in the pathogenesis of Asiatic cholera has not as yet been definitely established by direct evidence. The studies of Burnet and Stone(1) and those of Lam, Mandle and Goodner(2) suggest that this enzyme can induce intestinal damage such as may resemble the effects of cholera in man. However, the report by Singh and Ahuja(8) of mucinolytic activity in culture filtrates of vibrios isolated from non-cholera sources suggests that this enzyme may at best be only one of several

TABLE III. Antimucinase and Agglutinin Titers of Sera of Immunized Rabbits.

Immunizing antigens	Rabbit No.	Anti-mucinase titer	Agglutinin titer
None (pre-immunization sera)	1, 2	<20	<20
	4, 5	<20	<20
	7, 8	<20	<20
Mucinase solution*	1	640	1280
	2	320	320
	3	640	1280
Mucinase solution + cholera vaccine† (1:1)	4	320	640
	5	320	1280
	6	640	1280
Cholera vaccine	7	<20	1280
	8	<20	1280
	9	<20	1280

* Mucinase solution prepared from culture filtrate of *V. comma* strain 20A78.

† Lederle Cholera Vaccine, Lot No. 1976-67K.

factors involved in the disease process. If this proves to be the case, a truly effective cholera vaccine would have to contain a number of antigenic components. Jensen(3) has pointed out that the cholera mucinase must be maintained in its native state if it is to retain its antigenicity. Since the enzyme in the vibrio culture filtrate was extremely unstable, he suggested lyophilization as a method of preserving the *in vitro* activity and antigenicity of this material. The results of the present study, however, indicate that it is possible to obtain a stable mucinase solution, which can be combined with the present fluid cholera vaccine without altering the antibody-stimulating properties of the enzyme or of the somatic antigens.

No experimental data are available at this time to provide an explanation for the stability of the mucinolytic enzyme in the purified preparation as contrasted with the extreme lability of this enzyme in the culture filtrates. The purification procedure may have removed or inactivated some substance, perhaps another enzyme, which is responsible for the rapid deterioration of the mucinase. The possibility that one of the ingredients of the buffered calcium saline solution, employed as the solvent for the purified material, is protective must also be considered since Burnet and Stone found that the enzyme in culture filtrates diluted in buffered calcium saline is less readily inactivated by heat than in undiluted filtrates. Additional studies are planned to elucidate this point.

The partially purified mucinase solution has, in addition to its stability, other features which enhance its value as an immunizing antigen. The use of a dialyzed medium, which is not precipitated by ammonium sulfate, in the preparation of this material has eliminated high molecular weight medium constituents which might cause undesirable reactions when injected into animals or humans, and thus eliminates the necessity of developing chemical fractionation procedures for the removal of these substances. Furthermore, the preparation contains, in addition to the mucinase, other products of vibrio metabolism, some or all of which may be desirable components of an immunizing agent for Asiatic cholera.

Summary. 1. A method is described for the preparation from cholera culture filtrates of a mucinase solution which retains its *in vitro* mucinolytic activity for periods in excess of 2 years. 2. This preparation has been combined with a commercially prepared fluid cholera vaccine without affecting the *in vitro* activity of the enzyme, the antigenicity of the mucinase, or the antigenicity of the vibrio somatic substances. 3. The advantages of employing such a preparation as an immunizing agent for Asiatic cholera are discussed.

The technical assistance of Mr. Gerald A. Cole is gratefully acknowledged.

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1. Burnet, F. M., and Stone, J. D., *Austral. J. Exp. Biol. and Med. Sci.*, 1947, v25, 219.

2. Lam, G. T., Mandle, R. J., and Goodner, K., *J. Inf. Dis.*, 1955, v97, 268.

3. Jensen, K. E., *ibid.*, 1953, v93, 107.

4. Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sci.*, 1949, v27, 245.

5. Formal, S. B., and Lowenthal, J. P., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 10

6. Gottschalk, A., and Lind, P. E., *Brit. J. Exp. Path.*, 1949, v30, 85.

7. Lowenthal, J. P., unpublished data.

8. Singh, G., and Ahuja, M. L., *Indian J. Med. Res.*, 1953, v41, 285.

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Propagation of Measles Virus in a Strain of Human Epidermoid Cancer Cells (Hep-2).* (22677)

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In 1954 Enders and Peebles(1) reported the isolation of a cytopathogenic agent from patients with measles. Human and monkey kidney cultures were used in their study, but they reported that uninoculated monkey cultures may contain transmissible agents which cause effects at times difficult to distinguish from those of measles virus. The infectivity titer of human kidney cultures, including fluid and disrupted cells, was only $10^{3.5}$ TCD_{50} per ml. The purpose of this note is to report the adaptation of measles virus to a human cancer cell strain, Hep-2(2), and certain characteristics of the virus grown in this cell strain. Hep-2 is easily grown in the absence of human serum, has thus far proved free of latent agents, and can yield titers of measles virus greater than 10^6 TCD_{50} per ml.

Methods. Adaptation to Hep-2 cells. The 23rd human kidney passage of the Edmonston strain of measles virus† was inoculated into cultures of several cell strains, including Hep-2.† All cultures were kept in Eagle basal medium for HeLa cells(3) supplemented with 10% calf serum. The medium was changed every 4-7 days. No cytopathic (CP) changes characteristic of measles (multinucleate giant cells with intranuclear inclusions) were observed, but one HeLa culture degenerated in a nonspecific manner after 38 days incubation. When fluid from this culture was passaged into fresh HeLa and Hep-2 cultures, a non-specific type of degeneration again occurred in the HeLa culture but giant syncytial cells were found in Hep-2. Characteristic CP effects have been noted in later HeLa passages but they have been easier to recognize in Hep-2 cultures where the controls can be maintained in excellent condition for weeks in the medium free of human serum. CP changes have been observed in Hep-2 cultures 2 days after infection when large inocula were used but with small inocula 3-4 weeks might pass before recognizable changes appeared. Another measles virus line has been established in Hep-2 without HeLa passage, but after 2 passages in monkey kidney culture and with similarly prolonged initial incubation periods.

Results. Only 6 passages in Hep-2 cells have been made to date but multiplication has been established by the facts that, including changes, a total dilution of about 10^{46} of our original seed material has been involved and the titer of the last harvest was 100 times greater than that of the initial seed. The identity of the virus has been established by its characteristic CP effect and by a neutralization test. The typical CP effect was best manifest when the cultures were grown in Enders biological medium containing 35% Hanks salt solution, 35% beef amniotic fluid, 25% heated horse or calf serum and 5% beef embryo extract, or in a synthetic medium lacking glutamine(4). The neutralization test (Table I) was carried out by mixing 1/10 acute or convalescent serum from patients with measles, with serial virus dilutions and incubating one hour at room temperature before inoculation of Hep-2 cultures. Dr. John F. Enders had found that these patients had developed a marked rise in measles antibodies and kindly made aliquots of the sera available to us.

Growth of Virus in Hep-2 Cells. Cultures of Hep-2 cells in Eagle medium were inoculated with 10^5 TCD_{50} of adapted measles virus and washed 2 hours later. New virus was first detectable 18 hours after inoculation when the cells were ruptured by freezing and

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† The stock of measles virus and acute and convalescent sera were graciously supplied by Dr. John F. Enders of the Children's Hospital, Boston. The original stock of Hep-2 and of certain other cultures which were tested, were obtained through the kindness of Dr. Alice E. Moore of the Sloan-Kettering Institute, New York City.

TABLE I. Neutralization of Hep-2 Passage Measles by Human Serum.

Serum	Virus titer (neg. log.)	Logs virus neutralized
None	7.0	
Acute	7.2	0
Convalescent	2.5	4.5

TABLE II. Growth of Measles in Hep-2 Culture.

	Virus titer (neg. log.)	
Hr after inoculation	Frozen cells	Supernatant fluid
12	0	0
18	3.0	0
30	3.7	1.2
57	6.0	5.2

thawing. Free virus first appeared in the fluid about 12 hours later (Table II). Peak titers were achieved in 2½ days. The maximum complement-fixation titers have been ⅛. Enders and Peebles(1) reported that their human kidney grown material provided a usable antigen without concentration. It is thus apparent that Hep-2 cultures do not offer, in comparison with human kidney, proportionally as great increases in C-F antigens as of infective virus.

Infection of monkeys. Enders(5) has found the rhesus and cynomolgus macaques may possess naturally acquired antibodies against measles virus. In an attempt to determine how widespread this phenomenon might be, sera from 10 monkeys of various species have been tested. Six of these, including rhesus (*Macaca mulatta*), mangabey (*Cercocebus torquatus lunulatus*), green (*Cercopithecus aethiops sabeus*) and African white (*C. a. tantalus*), possessed virus inhibitors in their serum. The same sera, as well as 4 specimens from the African red grass monkey (*Erythrocebus patas*), gave positive C-F reactions with measles antigen. Eight monkeys, including 4 rhesus without pre-existing neutralizing antibodies, were inoculated intramuscularly or intravenously with 1 ml of Hep-2 passage virus. One of those with preantibodies had an elevated temperature and bouts of shivering for 4 days, beginning 12 days after injection. It is questionable whether this illness was associated with the inoculation. The only sign of illness in the other monkeys was a marginal temperature elevation in one monkey without antibodies 13 days after inoculation. Sera from 4 monkeys with antibodies and 2 without were checked for viremia. Positive results were obtained 7 and 13 days after inoculation from those monkeys without prior antibodies but not from the others. The virus that was isolated from the monkeys' blood did not cause recognizable CP changes in Hep-2 cells until after 30 to 56 days of incubation. It seems possible that the single monkey passage had partially reversed the adaptation of the virus to Hep-2 cultures.

Summary. Measles virus has been adapted to a cancer cell strain derived from a human epithelioma. The Hep-2 cell strain provides a particularly useful system for the study of measles because of its growth characteristics in media free of human serum, its freedom from latent cytopathogenic agents, and the high yields of virus that may be rapidly obtained. After adaptation to Hep-2 the virus was still capable of infecting monkeys but caused little or no illness.

The authors wish to express their gratitude to J. S. Huang for his contributions in the first phase of this work and to Stephen Greenwald and Sigrid Angerer for their technical assistance.

1. Enders, J. F., and Peebles, T. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 277.

2. Moore, A. E., Sabachewsky, L., and Toolan, H. W., *Cancer Res.*, 1955, v15, 598.

3. Eagle, H., *Science*, 1955, v122, 501.

4. Reissig, M., Black, F. L., and Melnick, J. L., *Virology*, in press.

5. Enders, J. F., *Am. J. Med. Sc.*, 1956, v231, 622.

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Hypoglycemic Action of Indole-3-Acetic Acid by Mouth in Patients with Diabetes Mellitus.* (22678)

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The administration of indole-3-acetic acid by mouth to normal rats results in a statistically highly significant hypoglycemia and a concomitant reduction in the rate of insulin destruction.† Since a similar decrease in the blood sugar does not occur in the severely diabetic alloxanized rat, the response cannot be due to an inhibition of the secretion or action of glucagon or to an acute non-specific hepatotoxic action. Accordingly the hypoglycemic response must be dependent upon the presence of insulin. This insulin dependence may be due to either an increase in the secretion or to a decrease in the rate of destruction of endogenous insulin consequent to an inhibition of the enzyme system, insulinase, which is relatively specific in catalyzing the hydrolysis of insulin. In accord is the fact that indole-3-acetic acid is an inhibitor of insulinase *in vitro* and *in vivo*.†

The present report deals with observations in man on the acute action of indole-3-acetic acid by mouth on the blood sugar concentration.

Method. Twelve adult patients with diabetes mellitus and 6 subjects without any evidences of a disturbance in carbohydrate metabolism were studied. In all instances, the patients with diabetes mellitus developed clinical evidences of the disorder after the age of 40 years (mean age of onset ± SE = 49.2 ± 2.0 years). The duration of the syndrome was from 2 to 16 years (mean duration ± SE = 7.7 ± 1.3 years). Those patients who were maintained on long-acting insulins were transferred to regular insulin for 3 to 5 days before the study in order to obviate the effect of insulin depots. All subjects served as their own controls in that the effect of drinking 5 ml of 0.5% sodium bicarbonate (pH 7.8)/kg body weight on the blood sugar concentration was studied on one occasion and the effect of a similar volume of bicarbonate containing 100 mg indole-3-acetic acid/kg body weight was determined on another occasion. The subjects were fasted overnight before each test and those with diabetes were not given any insulin on the morning of the test. Venous blood samples were taken before and at hourly intervals for 5 hours after the patients drank the solution and the concentration of glucose was determined by Nelson's method(1).

Results. A small, but statistically significant decrease in the blood sugar concentration occurred in the apparently healthy subjects after the ingestion of the indole-3-acetic acid dissolved in sodium bicarbonate (Fig. 1). Whereas the blood sugar concentration (mean ± SE) showed a slight increase from 82.7 ± 5.8 to 93.5 ± 5.8 mg % by the end of the 5 hours after the ingestion of the sodium bicarbonate, the blood sugar decreased from 81.7 ± 4.0 to 68.6 ± 2.7 mg % during the same interval after the indole-3-acetic acid solution. By the third hour the difference between the 2 responses was statistically highly significant ($P<0.001$).

The patients with diabetes mellitus likewise developed a hypoglycemic response to the indole-3-acetic acid (Fig. 2). Whereas these patients developed a maximum decrease in the blood sugar concentration from 257.3 ± 10.2 to 210.5 ± 13.3 mg % in 5 hours after the ingestion of the sodium bicarbonate solution, the blood sugar decreased from 232.9 ± 16.0 to 143.4 ± 11.8 mg % during the same interval after indole-3-acetic acid. The difference between the responses to the two solutions was statistically significant by the second hour ($P>0.01$) and thereafter. A repeated measures analysis of variance(2) likewise revealed a significant difference ($P>0.01$) between the curves of the blood sugar

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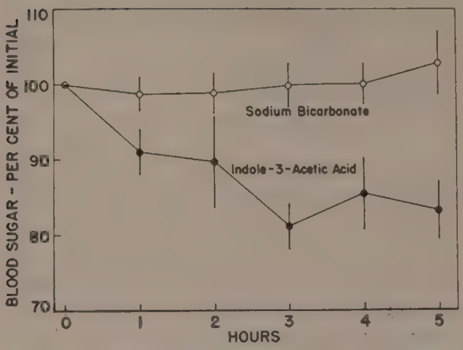

FIG. 1. Effect of indole-3-acetic acid on blood sugar concentration of non-diabetic subjects. Blood sugar concentration at each interval is expressed as the % (mean ± S.E.) of concentration prior to ingestion of the solution. Initial blood sugar concentration before the sodium bicarbonate solution was 82.7 ± 5.8 mg %, and 81.7 ± 4.0 mg % before the indole-3-acetic acid solution.

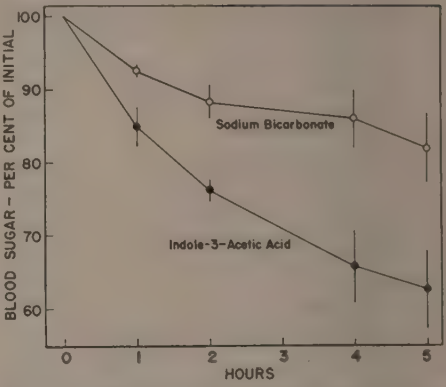

FIG. 2. Effect of indole-3-acetic acid on blood sugar concentration of patients with diabetes mellitus. Blood sugar concentration at each interval is expressed as % (mean ± S.E.) of concentration prior to ingestion of the solution. Initial blood sugar concentration before the sodium bicarbonate solution was 257.3 ± 10.2 mg %, and 232.9 ± 16.0 mg % before the indole-3-acetic acid solution.

responses to the two solutions.

Discussion. The relatively gradual decrease in the blood sugar concentration after the ingestion of indoleacetic acid by the non-diabetic subjects differs from the rapid maximal hypoglycemia which results within one hour after 1-butyl-3-p-tolylsulfonylurea by mouth(3). Various considerations have led to the hypothesis that the immediate hypoglycemic response of non-diabetic subjects to the tolylsulfonylurea is due to the discharge of insulin from the Islets of Langerhans into the circulation while the persistence of the hypoglycemia is due to the inhibition of insulinase and a consequent decrease in the rate of destruction of endogenous insulin(3). The response of the non-diabetic subjects to the indoleacetic acid, however, suggests that an acute stimulation of the Islets of Langerhans does not occur after the ingestion of this compound. Both the diabetic and non-diabetic groups show a gradual decrease in the blood sugar concentration which is similar to that observed in responsive patients with diabetes after the administration of tolylsulfonylurea (4) and may be attributed to an inhibition of insulinase and a decrease in the rate of insulin destruction. The data are in accord with the hypothesis that the insulin insufficiency of many patients with diabetes mellitus is due to an increase in the rate of destruction of insulin by the tissues rather than to a marked decrease in the rate of production of insulin by a severely damaged pancreas(5).

Although the data reported herein suggest that indoleacetic acid can be effective by mouth in decreasing the blood sugar of patients who developed diabetes after 40 years of age, the relatively small number of patients studied and the lack of information concerning the long-term toxic effects of indoleacetic acid and other plant growth hormones precludes their application to the therapy of diabetes mellitus at the present time. The studies, however, do suggest an hitherto unsuspected relationship between plant and animal hormones.

Conclusions. Indoleacetic acid by mouth produces a decrease in the blood sugar of adult patients with diabetes mellitus.

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1. Nelson, N., *J. Biol. Chem.*, 1954, v153, 375

2. Edwards, A. L., *Experimental Design in Psychological Research*, Rinehart and Co., N. Y., 1950.

3. Diengott, D., and Mirsky, I. A., *J. Pharm. Exp. Therap.*, in press.

4. Mirsky, I. A., Diengott, D., and Dolger, H., *Science*, 1956, v123, 583.

5. Mirsky, I. A., *Recent Progr. Hormone Research*, 1952, v7, 437.

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Effect of Castration upon Susceptibility to MEF$_1$ Poliomyelitis Virus Inoculated Intraperitoneally in Hamsters.* (22679)

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It has been shown that in comparison with normal hamsters inoculated intracerebrally with MEF$_1$ virus, the castrated animals showed a higher mortality from poliomyelitis while those treated with testosterone or gonadotrophic hormone showed a lower mortality(1). In order to determine whether castration is able to overcome the refractory state of hamsters to intraperitoneal inoculation of virus, the susceptibility to this route of inoculation was investigated at various periods following castration.

Materials and methods. Male Golden hamsters, 25-50 g, were used. A volume of 0.5 ml MEF$_1$ virus suspension diluted 1/50 in saline (*i.e.*, 800 Mouse LD$_{50}$) was inoculated intraperitoneally. Bilateral orchiectomy was performed at different intervals before and after inoculation of virus and infectivity in castrated animals was compared with that of non-castrated controls. All paralyzed animals were sacrificed and, in order to confirm the clinical diagnosis, their spinal cords were examined histologically and a suspension of the cord was inoculated into mice. Cortisone (1.5-2.5 mg) in a single intramuscular administration was injected for the study of individual and combined effects of castration and cortisone treatment. To investigate possible protective effects associated with compensatory phenomena following castration, the refractory state to intraperitoneal inoculation was eliminated by means of a single intramuscular injection of cortisone immediately prior to the viral inoculation. The results were analyzed by statistical procedures previously described(1).

Results. Seventeen % of castrated hamsters receiving MEF$_1$ virus intraperitoneally, 24 hours following orchiectomy, developed paralytic poliomyelitis, confirmed by mouse inoculation and by histological examination of the spinal cord. None of the similarly inoculated but uncastrated control animals developed the disease (Table I). When castration was performed 3 days after the administration of virus, only 1 animal out of 45 developed the disease, which may be explained by our previous observation of the rapid disappearance of intraperitoneally injected virus(2). Recent castration plus small doses of cortisone revealed a synergistic effect in enhancing susceptibility to poliomyelitis virus as compared to either castrated or cortisone treated controls alone (Table I).

The findings with respect to intraperitoneal inoculation of virus 14 days after orchiectomy were similar to those previously reported in hamsters inoculated intracerebrally where it was shown that compensatory phenomena usually occur at that stage following castration substituting a degree of resistance for the initial enhancement of susceptibility induced by testicular suppression(1). To determine whether a similar protective effect may be observed 14 days after castration in peritoneally inoculated animals, 2.5 mg of cortisone was administered intramuscularly followed by MEF$_1$ virus (800 mouse LD$_{50}$) inoculated intraperitoneally the same day. A significantly lower proportion of 14-day castrates developed the disease as compared to both 24-hour castrates and uncastrated controls receiving the same dose of cortisone (Table I).

Overdosage of testosterone significantly diminished the mortality of cortisone treated hamsters inoculated intraperitoneally with MEF$_1$ virus (Table II). However the reduction in paralytic incidence and mortality was of little statistical significance if the animals dying without paralytic symptoms were eliminated from both treated and control groups (Table II).

Discussion. From the above experiments it may be observed that orchiectomy is a conditioning factor in the pathogenesis of experi-

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TABLE I. Effects of Castration and of Cortisone Treatment upon Infectivity by Peritoneal Route in Hamsters. (All groups received a single intraperitoneal inoculation of MEF_1 virus, 800 mouse LD_{50}.)

Groups	Cortisone, mg	No. animals	Paralytic incidence, %	Significance of difference "P"
Non-castrated control	0	60	0	.001
24 hr after castration	0	35	17	
Idem	.5–1.0	58	21	.001
"	1.5–2.5	63	54	
Non-castrated control	1.5–2.5	105	36	.01
14 days after castration	2.5	29	10	

mental poliomyelitis since it enhances susceptibility to intracerebrally inoculated virus(1), and overcomes the refractory state to poliomyelitis virus inoculated peritoneally. With time, however, compensatory mechanisms come into play, and a state of resistance follows initial susceptibility induced by orchiectomy. This biphasic effect of castration raises the question as to whether susceptibility alterations are the result of suppression of testicular function or whether they are secondarily induced by the adrenal alterations that follow orchiectomy. It has been shown that during the initial 5 day period following castration, adrenal hypertrophy and involution of the thymus coincide with enhanced susceptibility to MEF_1 virus, while 14 days later, a progressive adrenal involution and thymus hypertrophy coincide with increased resistance to poliomyelitis(1). The fact that both adrenal hypertrophy and enhanced susceptibility to poliomyelitis follow any traumatic stress(2), suggests that during the initial stage following castration, enhancement of susceptibility was produced as a consequence of operatory trauma rather than by the suppression of testicular function. However, several other observations such as the inverse relationship between testicular maturity and susceptibility to poliomyelitis in relation to age and seasonal fluctuations(3), the protective effect of gonadotropine and of testosterone treatment(1), the association of large testes and resistance to poliomyelitis infection in certain races of hamster (Chinese, Albino), would indicate that testicular hormones have a protective influence against experimental poliomyelitis. Actually it is not possible to determine the individual contributions of adrenal gland and testis in determining the susceptibility level, since in addition to the possible direct hormonal interactions, the two endocrines influence each other indirectly through the mediation of the pituitary gland. Thus, orchiectomy produces polyphasic adrenal alterations which are followed by polyphasic fluctuations in susceptibility to poliomyelitis, while cortisone treatment produces testicular involution which is paralleled by enhancement of both morbidity and mortality from poliomyelitis(1). When cortisone treatment follows castration, their combined effects may be synergistic (initial stage), or

TABLE II. Effect of Testosterone Overdosage upon Mortality in Hamsters Injected with 3 mg Cortisone Intramuscularly and with MEF_1 Virus Intraperitoneally (800 Mouse LD_{50}).

		Total mortality (including animals dying without paralytic symptoms)		Mortality from poliomyelitis (excluding animals dying without paralytic symptoms)			
Preliminary treatment	No. of animals	Mortality, %	Significance "P"	Mortality, %	"P"	Paralysis, %	"P"
Depo–Testosterone* (15 mg i.m.)	249	43	.0002	29	.03	40	.04
Control	245	60		40		51	

* Upjohn Co.

antagonistic (compensatory stage), depending upon the experimental timing. From the effects of these interacting mechanisms it may be concluded that: refractoriness may be converted into susceptibility to poliomyelitis and vice versa, by factors influencing the adrenal-testis equilibrium either by direct action or by interference with pituitary function. The latter mechanism, which seems to be responsible for the effects of certain seasonal and dietary factors upon susceptibility(2,3), should be taken into consideration as one of the prospective means for disease prevention.

Summary. 1. A significant proportion of castrated male hamsters inoculated with MEF_1 virus intraperitoneally developed paralytic poliomyelitis, whereas non-castrated controls were completely refractory to the virus by this route. 2. Enhancement of susceptibility observed immediately after castration was found to be reversed during the compensatory stage 2 weeks later. 3. The effect of castration upon susceptibility to experimental poliomyelitis was synergistic with cortisone during the initial stage, but antagonistic during the compensatory stage.

1. Teodoru, C. V., and Shwartzman, G., *J. Exp. Med.*, 1954, v100, 563.

2. ———, Proc. Soc. Exp. Biol. and Med., 1956, v91, 181.

3. Shwartzman, G., Aronson, S. M., Teodoru, C. V., Adler, M., and Jahiel, R., *Ann. N. Y. Acad. Sci.*, 1955, v61, 869.

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Relationship between Behavioral Changes and Brain Cholinesterase Activity Following Graded Intracerebral Injections of DFP. (22680)

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Previous studies have shown that forced circus movements may be induced by unilateral electrostimulation(1,2,3,4) or ablation (5,6,7,8) of various parts of the neuraxis. Forced circling may also be produced by unilateral intracarotid injections of the anticholinesterase diisopropylfluorophosphate (DFP)(9,10,11,12) as well as by micrometer syringe injections of DFP and CNS stimulants into specific brain structures.[†] It has been disclosed that the intracerebral administration of 0.01 ml solutions containing from 0.06 to 0.16 mg of DFP does not result in significant spread of this substance to adjacent cerebral gray matter.[†] This finding opened the possibility to determine (1) the level of cholinesterase (ChE) activity at which stimulation of specific CNS structures occurs and (2) the relationships between the dose of DFP, the level of ChE activity and the production of forced circling. The caudate nucleus was selected for this study because of its comparatively high level of ChE activity(10,12) and the known role it plays in the forced circus movements induced by micrometer injections into this structure.[†]

Methods. In all experiments DFP was administered into the right caudate nucleus by means of a micrometer syringe attached to a stereotaxic apparatus. Solutions of DFP were made by diluting weighed amounts with water so that injections of 0.01 ml would deliver between 0.0001 mg to 0.32 mg. In order to inject greater doses undiluted quantities of DFP were employed because DFP is immiscible in this volume of water in amounts greater than 32 mg. The dose of undiluted DFP was determined by appropriate adjustment of the micrometer syringe and in all such cases the volume was less than 0.01 ml. The entire dose range of DFP employed was from 0.0001 mg to 10 mg. Controls were obtained by injections of 0.01 ml of physiological saline. Mock injections were also made in order to determine whether DFP was de-

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[†] In abstract, *Fed. Proc.*, 1956, v15, 199.

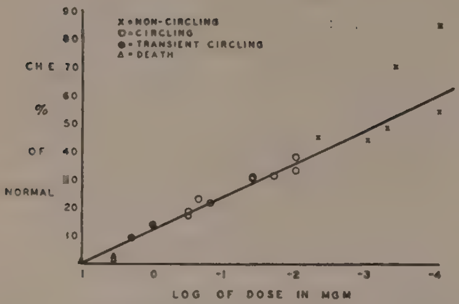

FIG. 1. Relationship between dose, % ChE activity and the behavioral response to inj. of from 0.0001 to 10 mg of DFP into the right caudate nucleus. Circling was always contraversive (*i.e.*, away from the inj. side). Note that one experimental value is shown at naught ChE activity. Also observe that 2 values for circling animals are almost superimposed.

posited in the brain merely upon insertion of the needle. To assure the delivery of solutions to exactly the point desired the bevel of a 27 gauge needle was ground off. This caused the direction taken by the drop to be parallel to the long axis of the needle. The needle was cleared with a stylus before each injection. Animals which had shown signs of bleeding during the experiment or at autopsy were discarded. Only male albino rabbits of approximately the same weight (average 2238 g) were selected for this study so that their cranial dimensions would be as uniform as possible, *e.g.*, the Bregma to Lambda skull length in all of these animals was close to 18.35 mm. A local anesthetic (0.5% pontocaine) was administered subcutaneously over the dorsum of the skull and the scalp was laid open. The animals were then lightly etherized and a hole drilled through the cranium with a dental burr 2 mm anterior to the transverse suture and 2 mm lateral to the longitudinal suture. The injection into the caudate nucleus was made at this point at a depth of 9 mm from the external skull surface. Ether administration was then stopped and the animal was released from the stereotaxic instrument and observed for at least 1 hr. The animals were then sacrificed by an intravenous injection of air and the ChE activities bilaterally determined in the caudate nuclei, dorsomedial thalami and motor cortices by the titrimetric method of Aprison, Nathan and Himwich(10). All ChE determinations were run "blind." In a second series of experiments 0.2 mg of atropine sulfate was administered with 0.16 mg of DFP and in a third one 1 mg of atropine sulfate was injected i.v. after forced circling was induced by the deposition of 0.16 mg of DFP into the nucleus caudatus.

Results. Injections of from 0.01 to 0.32 mg of DFP in solution into the caudate nucleus resulted in every case in contraversive forced circus movements which persisted for longer than 15 minutes (range from 18 min. to 1 hr. and 44 min.). As seen in Fig. 1 the ChE activity of the right caudate nucleus was below 40% of the control value in each of these circling animals. The ChE activity determinations on the left caudate nucleus, however, revealed values which were higher than 50% of normal and in general with doses of from 0.01 to 0.16 mg there was no significant evidence of spread of the injected DFP either to the opposite caudate or to the rest of the brain. With greater doses (0.23 to 0.32 mg) a spread of this substance throughout the brain was evident. Nevertheless the ChE activity values were always more than 50% of the control in parts of the brain other than the right nucleus caudatus. No other effects of DFP such as miosis or head nystagmus(9,11) were observed as a result of the intracerebral injection of this substance.

With doses of 1 and 2 mg of undiluted DFP contraversive circling was less than 8 minutes in duration and only intermittent. Doses of 3 and 10 mg did not cause circling and resulted in the death of the rabbit within 7 to 60 minutes after the intracerebral injections. In all of these animals the ChE activity in the left caudate nucleus was less than 35% of normal and in those that died it was near zero; the other brain areas in fatal cases had decreased to less than 13% of normal values.

On the other hand, injections of less than 0.01 mg of DFP did not cause any apparent change in the behavior of the animal and the ChE activity values were greater than 40% of normal. As shown in Fig. 1 the distribution of ChE activity of the right caudate nucleus in this noncircling group was greater than with the circling animals. No significant ChE activity reduction (less than 4%) resulted from the mock injections.

Addition of 0.2 mg of atropine sulfate to the injected solution of DFP prevented the appearance of forced circus movements. Moreover, the i.v. administration of 1 mg of atropine was effective in stopping the circus movements induced by previous intracerebral injections of DFP (0.04 to 0.16 mg).

Discussion. The results indicate that the optimum dose for the production of contraversive circling by intracerebral administration of DFP into the caudate nucleus is between 0.01 and 0.32 mg and that no significant spread of DFP from such injections may be expected with doses from 0.01 to 0.16 mg. Greater concentrations lead to an increasingly greater degree of spread of this substance, a finding which suggests that beyond a certain dose the quantity lost is increased progressively either by way of the circulation or cerebral spinal fluid. Because of this spread it was not possible to limit the reduction of ChE activity to a single brain structure to near zero by this method. Such low values for the caudate nucleus, however, have been reported when DFP is administered by means of the common carotid artery whereas other areas of the brain do not suffer a similar profound decrease in enzyme activity(10,13). Under these conditions electrographic(12,14) and other evidence(7) has been advanced to show that the nucleus caudatus is physiologically active. Since it was necessary to excise the entire caudate nucleus in order to obtain enough tissue for analysis, it is possible that the ChE activity of the caudate nucleus at the injection site was more reduced than reported in this study for circling animals. On the other hand, when DFP is administered intracarotidly the lenticulostriate vessels would cause a more uniform distribution of this substance to the caudate nucleus(12) and consequently an analysis of this whole structure may lead to lower values than was obtained by intracerebral injections.

This study also indicates that the ChE activity of one of the two caudate nuclei must be reduced to at least 40% of control value if circling is to be expected. There is no reason to believe that other brain areas need be affected by DFP to induce circling by intracerebral injections into this structure (see also reference†). The similarity between forced circus movements obtained in this study and those resulting from vestibular excitation with DFP(15) suggests a functional relationship between these two systems as proposed by Muskens(16). That death occurs when the total brain ChE activity is 10% of normal or less has been previously reported(17). Interestingly it occasionally took as long as an hour for massive doses of DFP to cause death when injected by micrometer syringe. This prolonged period would seem to offer a special advantage in the study of nerve gas antidotes when lethal doses are employed.

Atropine has been shown to correct the forced circus movements induced by DFP (injected via common carotid artery)(9) and it is thought therefore that DFP whether injected intracarotidly or intracerebrally has its effects by a cholinergic mechanism. The finding that intracerebral injections of DFP may induce circling in the rabbit supports the conclusions of other investigators that DFP administered intracarotidly causes circling through its central effects(13). None of the observable peripheral signs of the adversive syndrome(9,11) such as ipsiversive miosis are evident with intracerebral injections of DFP. The similarity between responses obtained with electrostimulation of different brain areas and micrometer syringe injections of DFP† reveal the possibility that this last procedure may be used as an additional method to study experimentally CNS cholinergic drugs and their antagonists.

Summary. The relationship between the amount of DFP injected into the right caudate nucleus and the resulting decreased cholinesterase activity has been investigated in regard to the appearance of forced circus movements. DFP in doses of less than 0.01 mg had no observable behavioral effect and did not lower the ChE activity to 40% of the control value. Doses of from 0.01 to 0.16

mg, however, caused persistent contraversive forced circling and decreased the cholinesterase value of the nucleus caudatus to from 21 to 40% of normal. There was no evidence of significant spread of the DFP within the brain in this group of animals. The administration of atropine sulfate either prevents or stopped these forced circus movements. Persistent forced circling could also be induced with larger doses of from 0.23 to 0.32 mg of DFP and evidence of spread of the DFP was then observed. In this group the injected nucleus caudatus showed ChE activity values of from 18 to 20% of the control whereas remaining parts of the brain revealed symmetrical reduction in ChE activity between 48 to 82% of normal. When still greater amounts of DFP were injected (1 to 10 mg) the spread of this substance progressively increased so that eventually ChE activity was decreased bilaterally and transient circling or death resulted.

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1. Anand, B. K., and Dua, S., *Science*, 1955, v122, 1139.

2. MacLean, P., *A.M.A. Arch. Neurol. Psychiat.*, 1955, v73, 130.

3. Mettler, F. A., *Res. Publ. Ass. nerv. ment. Dis.*, 1942, v21, 150.

4. Hinsey, J. C., Ranson, S. W., and Dixon, H. E., *Arch. Neurol. Psychiat.*, 1930, v24, 966.

5. Kennard, M. A., and Ectors, L., *J. Neurophysiol.*, 1938, v1, 45.

6. Mettler, F. A., and Mettler, C. C., *Brain*, 1942, v64, 242.

7. White, R. P., and Himwich, H. E., *Am. J. Physiol.*, 1954, v179, 684.

8. Lashley, K. S., *Brain*, 1921, v44, 252.

9. Freedman, A. M., and Himwich, H. E., *Am. J. Physiol.*, 1949, v156, 125.

10. Aprison, M. H., Nathan, P., and Himwich, H. E., *Am. J. Physiol.*, 1954, v177, 175.

11. Essig, C. F., Hampson, J. L., and Himwich, H. E., *Confinia Neurol.*, 1953, v13, 65.

12. Harwood, C. T., *Am. J. Physiol.*, 1954, v177, 171.

13. Essig, C. F., Hampson, J. L., McCauley, A., and Himwich, H. E., *J. Neurophysiol.*, 1950, v13, 269.

14. Hampson, J. L., Essig, C. F., McCauley, A., and Himwich, H. E., *EEG Clin. Neurophysiol.*, 1950, v2, 41.

15. Diamant, H., *Acta Oto-Laryngol.*, 1950, Suppl. 111, 84pp.

16. Muskens, L. J. J., *Brain*, 1922, v45, 454.

17. Nachmansohn, D., and Feld, E. A., *J. Biol. Chem.*, 1947, v171, 715.

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Sensitivity of *Staphylococcus aureus* to Penicillin in Various Media. (22681)

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The composition of the culture medium is of importance in testing the sensitivity of microorganisms to penicillin. The media may contain substances which react with penicillin chemically, thereby reducing its effective concentration. Substances having —SH groups such as thioglycollate, cysteine and glutathione behave in this manner(1,2,3,4). Acetic acid, too, inactivates penicillin by chemical interaction(5). Vitamins such as folic acid and vit. B_6 antagonise the effect of penicillin (6), and ascorbic acid also shows a deleterious effect(7). Glucose may diminish the effect of penicillin when added to cultures of *Staphylococcus aureus*(8,9). Furthermore, the activity of penicillin was decreased by addition of serum to the basal medium(10). On the other hand, certain substances, such as nicotinamide, exhibit synergistic effects with penicillin(8,11).

The present work deals with the effects of the addition of various carbohydrates to culture media, on the sensitivity of *Staph. aureus* to penicillin.

Methods. Test organism and inoculum: *Staphylococcus aureus* Oxford strain, was inoculated into nutrient broth (Difco). After 20 hours incubation at 37°C the cells were harvested and washed 3 times with physiological saline. The washed cells were diluted 1:100 in saline and 0.1 cc added to tubes containing 1 cc of medium. Two different media were employed: (a) Nutrient broth (Difco) and (b) a casein hydrolysate medium of the following composition:

Casein hydrolysate (vit. free)* 10%	3 cc
Carbohydrate	1000 mg
Tryptophan	20 mg
Cystine	10 mg
Thiamine	100 γ
Nicotinic acid	100 γ
Distilled water to make 100 cc	

After autoclaving, the following sterile solutions were added in amounts of 0.5 cc each: 1) Salt solution composed of: $MgSO_4 \cdot 7H_2O$ 4%; NaCl 0.125% $FeSO_4 \cdot 7H_2O$ 0.125%, and $MnSO_4 \cdot 4H_2O$ 0.125%. 2) K_2HPO_4 8.7%, and the medium was dispensed aseptically, into sterile tubes (12 x 100 mm).

Penicillin solution: Crystalline penicillin G (Shenley) was used. A concentrated solution containing 10,000 u/cc was kept in the refrigerator for one week; dilutions being prepared freshly for each experiment.

Antibacterial assay. The assays were carried out in small test tubes containing 1 cc of the medium. The penicillin effect was tested by the 2-fold dilution technic using the medium as a diluent for the penicillin. After inoculation with *Staph. aureus* the tubes were incubated at 37°C for various periods of time.

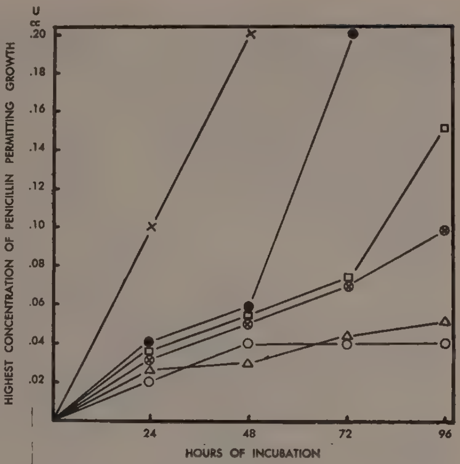

FIG. 1. Effect of carbohydrates on inhibition of *Staphylococcus aureus* by penicillin. Casein hydrolysate medium, —×—; casein hydrolysate medium with lactic acid (1%), —●—; casein hydrolysate with acetic acid (1%), —□—; casein hydrolysate with glucose (1%), —× (in circle)—; casein hydrolysate with pyruvic acid (1%), –△–; nutrient broth (Difco), —○—.

Results. Effect of different carbohydrates on the sensitivity of Staph. aureus to penicillin. Nutrient broth and casein hydrolysate media, containing glucose, pyruvic acid, acetic acid or lactic acid were inoculated with *Staph. aureus*. (Carbohydrates were added to the media before autoclaving.) Controls without added carbohydrate were included. Penicillin was added to the tubes in various concentrations, and the degrees of growth inhibition were examined after incubation for 24, 48, 72, and 96 hours. The results are given in Fig. 1, which shows that *Staph. aureus* was most sensitive to penicillin in nutrient broth and in a casein-hydrolysate medium containing pyruvic acid; 0.04 u/cc of penicillin inhibited the growth completely. The degree of inhibition did not change appreciably on further incubation. On the other hand, 0.2 u/cc of penicillin was not inhibitory when a casein hydrolysate medium without added carbohydrate was used. When lactic acid was added to casein hydrolysate, growth appeared in tubes containing 0.2 u/cc only after 72 hours of incubation. The growth of *Staph. aureus* in a medium containing acetic acid was not inhibited by concentrations of 0.15 u/cc after 96 hours. In Fig. 1 the sensitivity of *Staph. aureus* is plotted against incubation time. The straight line obtained in a glucose and penicillin-containing medium suggests the occurrence of a chemical interaction between them. To examine this point further, the following experiment was performed: penicillin in a concentration of 5,000 u/cc was incubated at 37°C in a solution of glucose (50 mg/cc). Penicillin incubated similarly in

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* Obtained from Nutritional Biochemicals Co. (NBC).

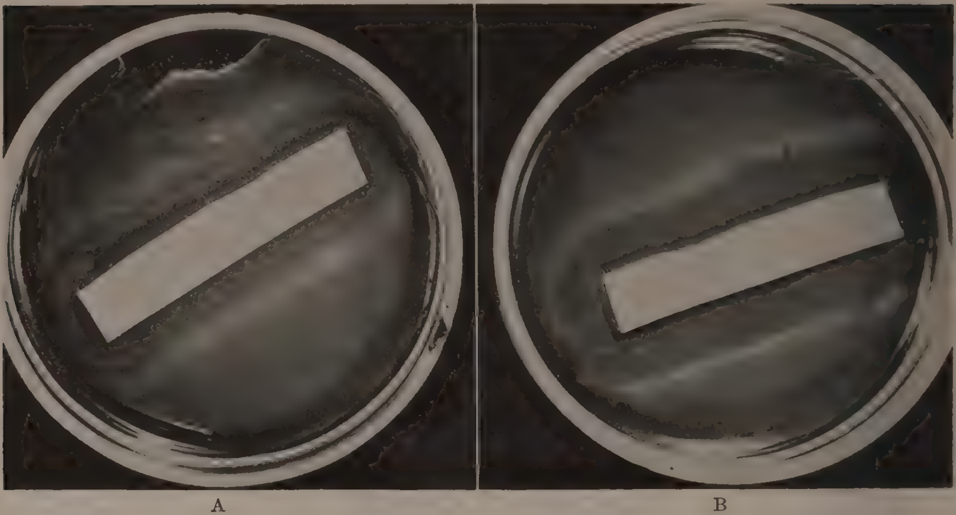

FIG. 2. Effect of glucose on inhibition of *Staphylococcus aureus* by penicillin. (A) Penicillin (5000 u/cc) was incubated with glucose (50 mg/cc) for 5 days at 37°C. Sterile filter paper strips were soaked with the solution and tested for activity against *Staphylococcus aureus*. Photographs taken after growth for 48 hr. (B) Penicillin (5000 u/cc) was incubated with saline solution for 5 days at 37°C. Sterile filter paper strips were soaked with the solution and tested for activity against *Staphylococcus aureus*. Photographs taken after growth for 48 hr.

saline served as a control. After 5 days, pieces of sterile filter paper were soaked in the respective solutions and were placed on agar plates inoculated with *Staph. aureus*. The results are shown in Fig. 2. It is seen that on incubation of penicillin with glucose a marked decrease of the inhibition zone was obtained. No diminution of penicillin activity was found after it was preincubated with lactate; it seems unlikely that a similar chemical reaction between penicillin and lactate had occurred. To eliminate the possibility that the addition of lactic acid stimulated the occurrence of a resistant mutant, the lactate-grown strain was inoculated into nutrient broth and its sensitivity to penicillin re-examined; however, no diminution in sensitivity was observed. Parallel experiments in which the media were sterilized by Seitz filtration were carried out. Table I shows that heating is not the cause of the variation in sensitivity in the different media.

Discussion. Pyruvic acid and glucose when added to media rich in amino acids (supplied as casein hydrolysate), increase the sensitivity of *Staph. aureus* to penicillin. On the other hand, lactic and acetic acids were not effective. The observed interaction between penicillin and glucose explains the reduction in penicillin activity after prolonged incubation in glucose containing medium. No such mechanism is responsible for the low activity of penicillin in lactic acid-medium. Potentiation of the penicillin activity by pyruvate may be explained as follows: (1) The antibiotic interferes with some step in the pyruvic acid metabolism of *Staph. aureus;* in the absence of added pyruvic acid such interference is less pronounced. Or (2) The toxic factor responsible for the antibacterial effect is *not* penicillin *per se* but a compound formed under its influence(12); pyruvic acid contributes to the production of the hypothetic toxic factor. In the absence of pyruvic acid the inhibitor is formed in smaller amounts, hence the lower antibacterial activity. Although the experiments presented do not favor one explanation rather than the other, unpublished work(13) suggests that penicillin interferes with the oxidation of pyruvic acid by cells of *Staph. aureus*. These results indicate that the sensitivity of *Staph. aureus* to

TABLE I. Sensitivity of *Staphylococcus aureus* to Penicillin in Casein Hydrolysate Media Containing Various Carbohydrates.*

	Acetic acid	Lactic acid	Pyruvic acid	Glucose	Nutrient broth	Casein hydrolysate, no carbohydrate (control)
Autoclaved medium	.15†	.2	.025	.1	.025	>.2
Seitz filtered medium	.15	.2	.05	.1	.025	>.2

* Results were read after 96 hr of incubation at 37°C.

† Maximal concentration of penicillin (u/cc) in which growth appeared.

penicillin may be affected by the carbon source of the medium. Similar findings were reported for *E. coli*(14) which was found to be more sensitive to penicillin in the presence of xylose or ribose than with glucose.

Summary. The effect of the composition of the medium on the sensitivity of *Staphylococcus aureus* to penicillin was tested. Pyruvic acid or glucose, when added to a casein hydrolysate medium, increase the antibacterial effect of the antibiotic. Lactic or acetic acids, on the other hand, have no such effect. The possible mechanisms of the enhanced penicillin activity in presence of pyruvic acid are discussed.

1. Chow, B. F., and Mckee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1945, v58, 175.

2. Geiger, W. B., and Conn, J. E., *J. Am. Chem. Soc.*, 1945, v67, 112.

3. Rinderknecht, H., *Pharmaceut. J.*, 1946, v156, 256.

4. Page, J. E., and Waller, J. G., *Nature*, 1946, v157, 838.

5. Florey, H. W., and Chain, E., *Antibiotic*, 1949, Oxford University Press.

6. Di Raimondo, F., *Int. Rev. Vitamin Research*, 1951, v23, 1.

7. Jalili, M. A., *Nature*, 1946, v153, 731.

8. Frieden, H. E., and Frazier, C. N., *Arch. Biochem. and Biophys.*, 1947, v15, 265.

9. Sykes, G., Lewis, D. G., and Goshawk, B., *Proc. Soc. Applied Bact.*, 1951, v14, 40.

10. Eagle, H., *J. Exp. Med.*, 1947, v85, 141.

11. Frieden, H. E., PROC. SOC. EXP. BIOL. AND MED., 1945, v60, 352.

12. Eagle, H., *J. Bact.*, 1951, v62, 663.

13. Bachrach, U., Ph.D. Thesis, 1953, The Hebrew University, Jerusalem.

14. Wainwright, S. D., and Mullaney, J., *J. Bact.*, 1954, v67, 504.

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Metabolic Studies of Desaminothyroxine. (22682)

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Desaminothyroxine (3,5 diiodo-4 (3′, 5′, diiodo-4 hydroxy phenoxy) phenyl-propionic acid) was synthesized by Clayton, *et al.*(1) at the Glaxo Laboratories, Middlesex, England, and by Kharasch *et al.** at the University of Southern California.

Bruice, Winzler and Kharasch(2) reported that in tadpoles (*Rana catesbiana*) at about 24°C, desaminothyroxine possesses 130 times the metamorphosing activity of L-thyroxine. Roth(3) has recently reported that it has 1000 times the activity of L-thyroxine in this test at 16°C. In comparison, Roche(4) has shown desaminothyroxine to have about 75% the antigoitrogenic activity of D-L-thyroxine in rats. Selenkow and Asper(5) reported it to have only 20 to 25% the antigoitrogenic activity of L-thyroxine. No reports on its calorigenic activity in mammals have been found.

Method. Measurements of the oxygen consumption of rats were conducted in a 5-unit basal metabolic rate machine at 30°C(6). Individual studies usually lasted 4½ hours, using the first half-hour as a calibration pe-

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* Personal communication.

TABLE I. Comparison of O_2 Consumption of Desaminothyroxine on Na L-Thyroxine.

Drug	Body wt, g	Initial body wt, baseline	O_2 consumption ($g\ O_2/100\ g$ rat/hr)			Avg increase O_2 consumption at 10 days ($g\ O_2/100 g$/rat/hr)
			3 days	7 days	10 days	
A. Dosage—.025 mg/day						
Desaminothyroxine	210 ± 50	.141	.155	.172	.186	.045
Na L-thyroxine	200 ± 30	.143	.201	.214	.218	.075
Controls	195 ± 25	.136	.127	.138	.144	
B. Dosage—.075 mg/day						
Desaminothyroxine	215 ± 55	.141	.161	.158	.194	.053
Na L-thyroxine	200 ± 30	.143	.230	.262	.275	.132
Controls	190 ± 10	.135	.135	.135	.143	

riod on rats fasted 24 hours. Four control runs were performed on each of 10 Long-Evans rats, weighing 160 to 230 g. The average oxygen consumption was .142 g O_2/100 g rat, per hour. The studies were carried out under conditions similar to those obtained by other investigators(8). Each animal acted as its own control and another control animal was evaluated with each group to check the variations of the machine. Four rats were injected intraperitoneally with coded Na-L-thyroxine, 0.025 mg/day; and four rats were given intraperitoneal injections of desaminothyroxine, 0.025 mg per day, in a double blind procedure. Individual studies were made at 3- to 4-day intervals for 2 weeks, reaching maximum oxygen consumption at 10 days. At this dosage the desaminothyroxine was found to be 60% as active as L-thyroxine, measured by the increase of oxygen consumption computed as grams of oxygen per 100 g of rat/hour. The 2 groups were then injected intraperitoneally with 0.075 mg daily, per rat, of desaminothyroxine, or L-thyroxine, for 2 weeks under the double-blind procedure. After 10 days on the tripled dosage, desaminothyroxine had only 40% the oxygen consumption of L-thyroxine. It was also noted that this drug produced only a 40% increase of oxygen above the control level, as compared to a 100% increase produced by L-thyroxine. (Table I).

Results. In trial studies at dosages of 0.15 and 0.30 mg daily/rat, desaminothyroxine was shown to have about 30% the calorigenic activity.

All animals returned to the pretreatment oxygen consumption levels after cessation of the drugs. No toxic signs or symptoms were noted during the one month course of drug administration, or in the one month following cessation of the medications. One rat died because of machine failure. The observation of Freinkel, *et al.*(9) of the marked absorption of thyroxine by glassware was confirmed. All drug containers were siliconized.

Discussion. The results, though variable with respect to the dosage, show that the metabolic effect of desaminothyroxine is of the same magnitude as that found in studies of its antigoitrogenic activity. In this respect the drug is less potent than L-thyroxine, but in comparison with the process of metamorphosis in amphibians, there is a wide variance (130 times more potency than Na L-thyroxine at 24°C(3) to 1000 times at 16°C)(4). These effects suggest the multiple activities of these drugs; perhaps separate or multiple metabolic pathways are involved.

Summary. Desaminothyroxine was shown to have 60% of the calorigenic activity of L-thyroxine at dosages of 0.025 mg/rat/day; 40% at the dosage of 0.075 mg/rat/day; and about 30% at dosages of 0.150 and 0.300 mg rat/day.

1. Clayton, J. C., Green, G. F. H., and Hems, B. A., *J. Chem. Soc.*, 1951, 2467.

2. Bruice, T. C., Winzler, R. S., and Kharasch, N., *J. Biol. Chem.*, 1954, v210, 1.

3. Roth, P. C. J., *Compt. rend. Soc. de biol.*, 1955, v149, 1180.

4. Roche, J., Michel, R., Wolf, W., and Etling, N., *Compt. rend. Soc. de biol.*, 1954, v148, 1738.

5. Selenko, H. A., and Asper, S. P., Jr., *Physiol. Rev.*, 1955, v35, 426.

6. Kleiber, M., *Univ. California Publ., Physiol.*,

1940, v8, 207.

7. Horst, K., *J. Nutrition,* 1934, v7, 277.

8. Farris, E. J., Griffith, J. Q., Jr., *The Rat in Laboratory Investigation,* 2nd Ed., J. B. Lippincott Co., 1949.

9. Freinkel, N., Dowling, J. T., and Ingbar, S. H., *J. Clin. Invest.,* 1955, v34, 1698.

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Inhibitory Effect of Chlorpromazine on Arterial Lipid Deposition in Cholesterol Fed Rabbits.* (22683)

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Reduced arterial lipid deposition in cholesterol fed rabbits has been achieved in a number of ways(1). Most of these inhibitory procedures cause weight loss, debilitation or other injurious effects. Chlorpromazine can be administered to animals for long periods of time without causing apparent impairment of health. Nevertheless, it is said to depress cellular activity, lower body temperature and arterial blood pressure, and, possibly, lower oxygen consumption of tissues(2). It is therefore reasonable to assume that this drug might alter cholesterol metabolism and influence the usual responses of rabbits to cholesterol feeding. The present experiment was designed to study the effect of chlorpromazine in this connection.

Method. Twenty-four albino rabbits weighing from 1810 to 2320 g initially were used. Nine received daily, 10 mg/kg intramuscular injections of chlorpromazine (Thorazine hydrochloride; Smith, Kline and French Laboratories)† and were fed rabbit food pellets containing 1% cholesterol(3). Nine rabbits were given equivalent injections of chlorpromazine but were fed the stock diet without added cholesterol. Six rabbits received only the cholesterol diet. The animals were given a daily ration of 100 g and food consumption was recorded daily. Body weights were determined every other week. Blood (9-10 cc) was drawn by ear vein initially and at the end of the 1st, 3rd, 6th, and 9th weeks. Free and esterified cholesterol, phospholipid, total lipid, proteins and sugar were determined on the serum as described previously(3). Determinations of serum bilirubin (Evelyn and Malloy) were also attempted but the levels were always too low to obtain quantitative readings. All animals survived and were sacrificed at the end of the tenth week. Complete gross and microscopic studies of the viscera were performed. The aortas were stained grossly with Sudan IV and the distribution of lipid plaques charted as previously described(3).

Results. Blood chemistry: Cholesterol levels rose progressively in all cholesterol fed animals but by the end of the third week it was apparent that the chlorpromazine group had significantly lower values (Table I). At the ninth week the total cholesterol of this group was only about one-half as high (Mean 985, Max. 1368, Min. 760 mg/%) as that of those fed the 1% cholesterol diet only (Mean 1834, Max. 2166, Min. 1535 mg/%). The ratio of free to esterified cholesterol did not change significantly in either group. Total cholesterol rose slightly and transiently in 8 of 9 chlorpromazine rabbits on stock diet reaching its peak during the third week. In these 8 rabbits the mean cholesterol was 322 mg/% (Max. 457, Min. 234). No explanation can be given for the failure of the 9th rabbit to show this change. It fell in all 8 to a mean level of 157 mg/% by the sixth week and was within normal limits at the ninth week. The free and ester fraction ratios were not altered during this temporary rise.

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† We wish to thank the Smith, Kline & French Laboratories for donating this preparation.

TABLE I. Total Serum Cholesterol in Cholesterol Fed and Chlorpromazine Treated Rabbits.

	Total serum cholesterol, mean mg %				
	Wk of exp.				
	0	1	3	6	9
Chlorpromazine only	61	68	293	157	95
Chlorpromazine and cholesterol	55	411	759	961	985
Cholesterol only	65	374	1140	1560	1834

Serum phospholipids (Table II) were also elevated in all cholesterol fed animals but to a lesser degree than serum cholesterol. The ratio of cholesterol to phospholipid was very similar in both the chlorpromazine and non-chlorpromazine treated, cholesterol fed rabbits. It varied from about 3:1 to 2:1 in most determinations. Phospholipid values were also slightly elevated in the third and sixth weeks in rabbits receiving only chlorpromazine but generally lagged behind the slightly elevated serum cholesterol. There was, however, no significant change in the ratio of these 2 lipid fractions. Neutral fat (calculated as previously described(4)) was slightly elevated in all cholesterol fed animals especially in the latter weeks of the experiment, varying from 0.48 g % to 0.82 g %. There was no significant difference between the chlorpromazine-treated and non-chlorpromazine treated cholesterol fed groups. Neutral fat levels remained low and remarkably constant throughout the experiment in the group receiving only chlorpromazine. Serum globulin tended to rise in all chlorpromazine treated rabbits but this was inconstant in degree. A few had reversed albumin globulin ratios on several occasions. Blood sugar and serum bilirubin were never significantly elevated.

Body weight and food consumption. All 24 rabbits consumed their entire daily 100 g food ration except on very few occasions when 10-20 g of food was left in the containers. None developed diarrhea. All animals gained weight consistently throughout the experiment. The mean weight gain in the group receiving both chlorpromazine and cholesterol was significantly less (58 g/wk) than in the groups receiving only chlorpromazine (103 g/wk) and cholesterol only (98 g/wk). All animals were in good health throughout the experiment and after the first 2 injections of chlorpromazine showed no obvious signs of sedation. Body temperature as measured intraperitoneally with a telethermometer during the eighth week of the experiment was not lowered significantly in chlorpromazine treated rabbits.

Necropsy findings. A striking inhibition of lipid deposition in the aortas, heart valves, and pulmonary arteries was noted in all but one of the cholesterol fed animals that received chlorpromazine (Fig. 1). In these 8 only a few thin patches of lipid were found chiefly in the aortic arch. One rabbit had fairly extensive plaques, but even in this animal the lesions were less extensive than in all but one of the group receiving cholesterol only. Such individual variation is a common finding in cholesterol feeding experiments in rabbits. Rabbits receiving chlorpromazine only had no gross vascular disease.

The livers of the rabbits receiving both chlorpromazine and cholesterol were smaller (mean wt 103 g) and less yellow than those that received cholesterol only (mean wt 125 g). They were only slightly larger and paler than the livers of animals that received only chlorpromazine (mean wt 95 g). Histologically, the liver cells of the chlorpromazine

TABLE II. Serum Phospholipid and Neutral Fat in Cholesterol Fed and Chlorpromazine Treated Rabbits.

		Wk of exp.				
Group		0	1	3	6	9
Chlorpromazine only	Phospholipid (mean mg %)	109	74	175	141	91
	Neutral fat (mean g %)	.35	.39	.45	.38	.42
Chlorpromazine and cholesterol	Phospholipid (mean mg %)	51	277	445	377	431
	Neutral fat (mean g %)	.38	.48	.60	.55	.55
Cholesterol only	Phospholipid (mean mg %)	67	210	496	600	771
	Neutral fat (mean g %)	.38	.43	.27	.61	.82

treated, cholesterol fed rabbits were less swollen and contained less finely dispersed lipid droplets than did those of the cholesterol fed animals that received no chlorpromazine. No evidence of liver cell damage or bile stasis was noted in any chlorpromazine treated rabbits. Enlargement and increased lipid storage was equally prominent in the adrenal cortices of both cholesterol fed groups. Histologic study of the other viscera revealed no significant changes that could be attributed to chlorpromazine administration. The mean heart weight of all 3 groups was approximately the same.

Discussion. It seems likely that the reduced amount of lipid deposited in the arterial walls in chlorpromazine treated, cholesterol fed rabbits is related to the lower levels of blood cholesterol observed in these animals as compared to the cholesterol fed rabbits that did not receive this drug. Except under special circumstances the degree of arterial lipid deposition in cholesterol fed rabbits varies directly with the degree of hypercholesterolemia in our own experience. There is no evidence that alterations in blood lipid interrelationships such as lowered cholesterol/phospholipid or cholesterol/neutral fat ratios played any role. It is also clear that malnutrition or general debilitation were not a factor in inhibiting arterial lipid deposition since the chlorpromazine treated animals remained in good health throughout the experiment. The degree of inhibition of lipid deposition was greater than that observed previously(3) in cortisone treated, cholesterol fed rabbits on a comparable regimen. If generalized depression of metabolism was involved such as occurs in hypothyroidism it might be expected that chlorpromazine would have accentuated hypercholesterolemia and enhanced arterial lipid deposition. This was obviously not the case. The failure of chlorpromazine treated, cholesterol fed rabbits to gain as much weight as the control groups, suggests the possibility that even though these animals consumed as much cholesterol, its absorption from the gut may have been reduced. However, the mean weight gain in the rabbits receiving only chlorpromazine was as great as in those fed cholesterol only.

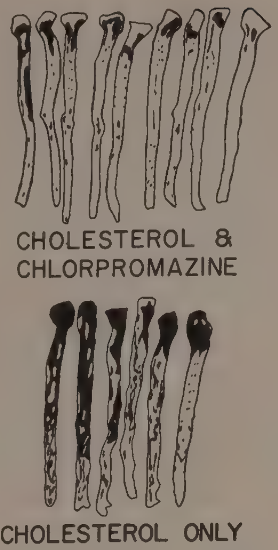

FIG. 1. Extent of aortic lipid deposition in rabbits fed cholesterol and inj. with chlorpromazine (upper row) compared with that in rabbits fed cholesterol only (lower row). Black areas represent portions of the intima covered by sudanophilic lipid deposits.

The inhibitory effect of chlorpromazine on hypercholesterolemia in these rabbits may have been mediated through the liver. Blood cholesterol levels are believed to be closely regulated by the metabolism of this lipid in the liver(5). To support this interpretation is the finding that the livers of the chlorpromazine treated animals failed to enlarge and to store as much lipid material as did those of rabbits that received only cholesterol. Chlorpromazine is known to produce jaundice occasionally and presumably some sort of liver injury in man although the exact mechanism has not been elucidated. The transient rise in serum lipids and globulin during the 3rd and 5th weeks in rabbits receiving only chlorpromazine was greater than ordinarily occurs in untreated rabbits and was therefore presumably due to the drug. These changes suggest that a brief period of metabolic readjustment may have occurred. This period corresponds somewhat to the time at which jaundice appears most frequently in man after chlorpromazine therapy is instituted. In some instances these attacks of jaundice in man have been associated with moderate elevations of serum cholesterol(6). This has usually been explained on the basis of biliary stasis. There appears to be little evidence that chlorpromazine has any striking effect on the usual blood lipid levels found in man. Hollister and Kanter(7), however, report that it may be effective in lowering blood lipid levels of patients with essential hyperlipemia.

Summary. The blood cholesterol levels of cholesterol-fed rabbits given daily intramuscular injections of chlorpromazine fail to rise as high as in rabbits fed cholesterol only. Deposition of lipid in arteries and in the liver is also greatly reduced. Repeated injections of chlorpromazine in rabbits on stock diets may cause transient periods of mild hypercholesterolemia.

1. Katz, L. N., and Stamler, J., *Experimental Atherosclerosis*, Charles C. Thomas, Springfield, Ill., 1953.

2. Dundee, J. W., *Brit. J. Anesthesia*, 1954, v26, 357.

3. Stumpf, H. H., and Wilens, S. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 219.

4. Wilens, S. L., and Stumpf, H. H., *Am. J. Path.*, 1955, v31, 275.

5. Gould R. G., *Symposium on Atherosclerosis*, Publication 338, p153, *Nat. Acad. Sci.—Nat. and Res. Council*, Washington, 1954.

6. Moritt, E. R., Meyer, M. A., Snell, A. M., Goldman, M. J., Gibson, J. R., Sullivan, B. H., Webster, J. G., and Stone, R. B., *Gastroenterology*, 1955, v28, 901.

7. Hollister, L. E., and Kanter, B. S., *ibid.*, 1955, v28, 1069.

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Studies with 5-Bromouracil in Rodents and Dogs.* (22684)

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The growth inhibitory activity of 5-bromouracil was first observed in *Lactobacillus casei*(1). Later the substance was shown to be extensively incorporated in lieu of thymine in the deoxyribonucleic acids of bacteria(2, 3,4) and of a T_2 phage of *Escherichia coli*(4). Such findings suggest that the agent may be a thymine antagonist—a supposition supported by the near identity in size of the bromine constituent and of the methyl group of thymine (2). Anti-thymine actions may also be involved in the potentiation by 5-bromouracil of the inhibitory effects of folic acid antagonists in *L. casei*(5). Because of these findings, it has become of interest to ascertain whether 5-bromouracil might potentiate the action of amethopterin in the treatment of human leukemia.† With this in mind the work presented here was initiated as a preclinical study of the pharmacological actions of 5-bromouracil. During the study special interest arose in its catabolic fate *in vivo* because of the presence of crystalline deposits in kidneys or bladders of intoxicated animals. It is the primary purpose of this report to document this finding and to present information concerning the chemical nature of these deposits.

Procedure. Mice, rats and dogs were the subjects of the present investigation. The

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The 5-Bromouracil was purchased from the Dougherty Chemical Co., New York City.

† Personal communication from Dr. J. H. Burchenal.

TABLE I. Toxicity of 5-Bromouracil in Rodents.

Species	Administration	No. of successive daily doses	LD_{50}* (mg/kg/day)	19/20 confidence limits* (mg/kg/day)	S*
Mice	Intraper.	1	1400	1120–1750	1.25
	”	5	700	†	1.13
Rats	Intraper.	1	1700	1420–2040	1.16
	”	5	710	530– 960	1.42
	Oral	5	1410	1040–1900	1.42

* Calculated according to Litchfield and Wilcoxon(11). All animals were observed for 14 days after end of treatment.

† Confidence limits not calculated since mortality for doses used was either >84% or <16% (11).

mice in all cases were male Swiss albino mice (Millerton Research Farms). Male CFW (Carworth Farms Wistar) rats were employed for the toxicological studies while the pathological studies were carried out with the male CFN rats (Carworth Farms Nelson). The latter are a sub-strain of Wistar rat which has been bred free of "chronic murine pneumonia," salmonellosis and bartonellosis (6,7). They tolerate larger doses of most agents which we have recently tested than do CFW rats. Most of the toxicological, hematological and biochemical methods employed have been described (8,9). The 5-bromouracil was administered as suspensions in 0.9% NaCl containing 0.5% CMC (carboxymethylcellulose). The mice and rats received 1 ml of suspension per 100 g of body weight given either intraperitoneally or by stomach tube. The total daily oral dose for each dog was suspended in 50 ml of the saline CMC solution and administered by stomach tube using 50 ml of water to rinse the tube. For intravenous administration in dogs the agent was solubilized in 0.9% NaCl by the addition of molar equivalent amounts of ethanolamine. Because of limitations of solubility it was not practical to give intravenous doses greater than 200 mg/kg/day. Plasma and serum bromide levels were determined by the method of Brodie and Friedman(10). When 5-bromouracil was added to plasma or serum this method did not give rise to the liberation of bromide ion.

Results. Course of intoxication in rodents. Table I presents toxicity studies of 5-bromouracil in mice and rats. The toxicity of this agent was similar in both species. Single lethal doses, 2 g/kg, caused death within 24 hours. Following such doses the animals became weak, dyspneic, and depressed within 2-3 hours and the depression increased progressively until death. Several hours after the injections some animals had red tears and a few showed an orange urine. In animals treated with successive daily doses whether by the intraperitoneal or oral route, depression was noted several hours after each administration of the higher doses (1000 mg/kg/day intraperitoneally or 2000 mg/kg/day by mouth.) Deaths occurred between the second and seventh days in these groups. Ruffled fur was also prominent at 2 to 4 days in the animals given 500 to 1000 mg/kg/day and red-brown lacrymation as well as deep orange urine was observed. Two weeks after the last of these doses the survivors appeared healthy and the experiment was discontinued. At lower doses rodents showed no signs of intoxication. As will be seen below, the administration of 5-bromouracil gives rise to significant concentrations of bromide ion in plasma. This suggested a possible explanation for the depression noted in the treated animals. To test this possibility 2 groups of rats (6 rats/group) were given single, intraperitoneal injections of molar equivalent amounts of bromine as 5-bromouracil and NaBr, 2 g/kg and 1.08 g/kg, respectively. The 5-bromouracil animals died within 24 hours whereas the animals receiving sodium bromide recovered after a period of depression. At one-half of these doses both groups of 6 animals survived with no overt signs of intoxication. These results indicated that the liberation of bromide *in vivo* cannot be solely

TABLE II. Chromatographic and Spectral Findings.

	R_f	Maxima		Minima	
		pH 5	pH 13	pH 5	pH 13
Uracil standard	.25	259	284	228	242
5-bromouracil standard	.37	274	289, 226	247	258
A. Rat kidney deposits	.40	275	290, 226	243	255
B. Rat urine spot 1	.26	259	283	228	242
2	.39	274	289, 225	244	256
C. Dog bladder deposits	.40	275	290, 226	242	256

responsible for the acute deaths caused by 5-bromouracil.

Pathological changes in rats. As mentioned above, pathological changes were studied in CFN rats. For this purpose, 18 rats were given 2 g/kg/day by oral intubation for 4 consecutive days. Six rats, chosen at random, were sacrificed at 96 hours (that is, at 24 hours after the last dose). At this time the average weight loss was 15%. The remaining animals, which had also lost weight, were observed for an additional 2 weeks; at the end of this period they had regained their weight and appeared healthy. In all the sacrificed rats the spleen appeared small. In 2 of the animals, congestion and hemorrhage were noted in the cervical lymph nodes. The kidneys of all were enlarged and, when transected, the cortices appeared edematous. Yellowish-gray crystalline deposits encrusting the papilla were found in the pelvis of each kidney. The bladders were filled with bright red urine. The principal microscopic changes were observed in the urinary tract. In all animals an internal hydronephrosis was present associated with small foci of acute pyelonephritis. Only rare tubular deposits were observed in the papillas. These were pink amorphous masses which only rarely contained needle-like structures. Since many crystalline masses were observed grossly, the paucity of deposits observed microscopically may be accounted for by loss during histological processing. More often rounded bodies which stained dark blue were seen in the medulla. These had the appearance of calcospherites. The basement membranes of all glomerular capillaries were slightly and diffusely thickened. Most of the convoluted tubules contained protein casts. A mild cystitis was present in the bladder associated with edema of the wall; edema was also observed in the prostate. In one animal pink masses similar to those observed in the papilla were present in the lumen of the bladder. In the spleens the red pulp appeared to contain fewer nucleated elements than in control rats. Sections of sternum revealed marked congestion of the marrow. In 5 animals there was a slight decrease of nucleated elements which varied from 10 to 20%. In 1 animal the decrease was 50%. The remaining tissues including lymph nodes, intestines and liver showed no significant changes. The kidney deposits from 2 of the rats mentioned above were removed with a spatula and saturated aqueous solutions were prepared. Descending paper chromatograms were developed with these solutions using Whatman No. 3 MM paper and n-butanol saturated with water. About 20 γ of material was applied to each spot at the origin. After development of the chromatograms, the ultraviolet absorbing components were visualized by means of a 15 watt General Electric Sterilamp equipped with a Corning No. 8963 filter. In this case, as in all chromatographic studies reported herein, uracil and 5-bromouracil[‡] standards were used. The ultraviolet absorbing spots were eluted and read against appropriate blanks at pH 5 and pH 13. The results are shown in Table IIA. The R_f as well as the spectrum in water and alkali indicated that for the most part the crystalline material was unchanged 5-bromouracil.

Metabolic fate in rats. Additional studies were made of the fate of 5-bromouracil in rats (CFW). Six animals were placed in individual metabolism cages without food but with free access to 0.9% NaCl in drinking bottles and were starved for 18 hours prior to and during the experimental period. Three received intraperitoneally 0.5% CMC in saline and the other 3 1 g/kg of 5-bromouracil. The 24-hour urine from each rat was collected and diluted with 2 parts water. Three ml portions were then evaporated to 0.2 ml and the concentrated urines were chromatogrammed as described above. Little or no ultraviolet absorbing material was noted in the control urines. Two distinct spots (No. 1 and No. 2—Table IIB) were observed on the chromatograms of the urines of rats receiving 1 g/kg. From the R_f of these spots and from their absorption spectra they corresponded to uracil and unchanged 5-bromouracil, respectively. No attempt was made to determine quantitatively the amounts nor ratio of the components present. It should be noted that plasma bromide levels at this time were 26-27 mg/100 ml in the treated animals as compared to 0 in the control rats. Since bromide ion is considered to be distributed throughout the extracellular fluid, that is, in about 25% of body weight, this plasma level would have resulted from the dehalogenation of about 12% of the 5-bromouracil injected. This probably represents the minimum amount of 5-bromouracil which was converted *in vivo* to uracil.

[‡] The 5-bromouracil standard employed was the same as that used in the animal studies. It contained no discernible uracil (Table II).

Course of intoxication in dogs. All animals were given a maximum of 10 successive daily doses excepting weekends and were observed for 2 weeks following the last dose. Intravenous doses of 100 and 200 mg/kg/day in 2 respective pairs were well tolerated and the animals showed no untoward signs. Two dogs which received 1 g/kg/day by mouth as well as 1 of 2 dogs which received 0.5 g/kg/day died during or shortly after treatment ended, that is, at 6, 14, and 16 days, respectively. Anorexia was noted at 2 to 6 days prior to death in these 3 dogs. Other signs of intoxication were weight loss and diarrhea. One of a pair of dogs receiving 250 mg/kg/day orally showed a transient anorexia and weight loss but both of this pair survived and were healthy at the termination of the experiment.

To determine the nature of lesions associated with intoxication, 2 dogs were given 1 g/kg/day orally for 4 consecutive days and sacrificed 24 hours after the last dose. At this time biochemical and hematological analyses revealed no significant changes from pretreatment values in the following: glucose, clotting time and bromosulfalein retention in blood; chloride, protein and non-protein nitrogen in plasma; hematocrit, total and differential counts of leucocytes and counts of reticulocytes in blood; and counts of nucleated cells in aspirates of bone marrow from the iliac crest. There was present, however, in the serum of both dogs a high concentration of bromide: 62 mg/100 ml. One of the pair presented a normal picture at autopsy. In the other dog the bladder was found to contain dark red urine which was positive for blood. The platelet count in the blood of this animal had decreased from the initial value of 140 x 10^3/mm^3 to 7 x 10^3/mm^3 at time of sacrifice. This dog's bladder was also filled with a dark tan solid mass. Its kidneys appeared normal.

The solid mass was removed from the bladder for further study and the findings are summarized in Table IIC. The chromatographic and spectral studies showed that the only detectable ultraviolet absorbing material present was 5-bromouracil.

In the same animal the only significant lesion observed microscopically was the presence of a few small mucosal hemorrhages in the wall of the bladder. Sternal and vertebral bone marrow, lymphoid tissues and intestine as well as all other tissues were normal. Neither crystals, hydronephrosis nor pyelonephritis were observed in the kidneys. In the other dog no microscopic changes were present in *any* of the tissues.

Discussion. It has been reported that 5-iodo-2-thiouracil is converted to urinary thiouracil in the dog(12) and is de-iodinated in the human(13). While this manuscript was in preparation Barrett and West(14) reported a study of a variety of halogenated pyrimidines in the rat. They found that parenteral administration of 5-bromouracil (40 mg/rat x 1) led to the urinary excretion of uracil which was identified by its behavior in several paper chromatographic systems.

In the present study, it was found that oral administration of 5-bromouracil (1 g/kg x 1) to the rat gave rise to the excretion of both uracil and 5-bromouracil. The urinary components were studied by spectrophotometric as well as paper chromatographic technics. It was of interest to note a concomitant rise in plasma bromide levels. It was also of interest to note that when 5-bromouracil was given in high repetitive doses to rats and dogs, the foreign deposits which appeared in the kidney of the former and in the bladder of the latter were composed predominantly of unchanged 5-bromouracil. Although crystals were found in only one of the 2 dogs sacrificed, the serum bromide levels in both dogs were equally elevated. Presumably *in vivo* dehalogenation readily occurs in this species. The present investigation has revealed no clear-cut evidence regarding the cause of death in dogs given 5-bromouracil. It may be suggested that crystallization of the agent in the urinary tract and bromide poisoning may both contribute to intoxication. If this agent is to be considered feasible for clinical trial there is a real danger of the development of renal insufficiency or bromism or both as judged from the present findings which were consistent in both rats and dogs.

It should be pointed out that although microbiological evidence indicates that 5-bromouracil interferes with nucleic acid synthesis, lesions were not found in the bone marrow or intestinal epithelium of dogs given toxic doses. Only minimal changes in bone marrow were observed in rats given high doses of 5-bromouracil which were without discernible effect in the intestine of this species. This is in contrast to other agents, such as 6-mercaptopurine(8), which are believed to interfere with nucleic acid synthesis and which have primary sites of action in proliferating tissues such as the bone marrow and the intestine.

Summary. The toxicity of 5-bromouracil has been studied in mice, rats and dogs. Single lethal doses in rodents caused death within 12 to 24 hours. Somewhat smaller amounts when given repeatedly in daily doses gave rise to crystals in the kidney. These have been shown by chromatographic and spectrophotometric methods to be predominantly unchanged 5-bromouracil. The urines of rats given a single high dose of the compound have been found to contain uracil and 5-bromouracil. In dogs deaths are produced by the oral administration of 0.5 to 1.0 g/kg daily excepting weekends for a total of 10 doses. The principal changes noted in fatally intoxicated dogs were weight loss, anorexia, diarrhea and significant concentrations of bromide ion in plasma. In one such animal, the bladder with filled with a deposit of 5-bromouracil.

1. Hitchings, G. H., Falco, E. A., and Sherwood, M. B., *Science*, 1945, v102, 251.

2. Weygand, F., Wacker, A., and Dellweg, H. Z., *Naturforch*, 1952, v7B: 19.

3. Zamenhof, S., and Griboff, G., *Nature*, 1954, v174, 307.

4. Dunn, D. B., and Smith, J. B., *ibid.*, 1954, v174, 305.

5. Elion, G. B., Singer, S., and Hitchings, G. H., *J. Biol. Chem.*, 1954, v208, 477.

6. Cummings, C. N. W., *Carworth Farms Quart. Letter* 1956, no. 40, New City, Rockland County, N. Y.

7. Nelson, J. B., *J. Exp. Med.*, 1951, v94, 377.

8. Clarke, D. A., Philips, F. S., Sternberg, S. S., Stock, C. C., Elion, G. B., and Hitchings, G. H., *Cancer Research*, 1953, v13, 593.

9. Philips, F. S., Sternberg, S. S., Hamilton, L. D., and Clarke, D. A., *Cancer*, in press.

10. Brodie, B. B., and Friedman, M. M., *J. Biol. Chem.*, 1938, v124, 511.

11. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharm. Exp. Therap.*, 1949, v95, 99.

12. Plummer, A. J., *Fed. Proc.*, 1950, v9, 309.

13. Klein, E. M., *Klin. Wochschr.*, 1953, v31, 17.

14. Barrett, H. W., and West, R. A., *J. Am. Chem. Soc.*, 1956, v78, 1612.

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Cystine Requirement for Normal Poliovirus Action on Monkey Kidney Tissue Cultures.* (22685)

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With the development of medium 199 Morgan, Morton, and Parker(1) provided a chemically defined medium which has been found to maintain monkey kidney cells in tissue culture during viral action. More recently, Eagle(2) studied the growth requirements of HeLa cells and mouse L cells and devised for these 2 cell types growth media which are chemically defined except for nondialysable serum components. At this laboratory Eagle's medium for HeLa cells without serum has been found to be adequate not only for the maintenance of monkey kidney cells but also for poliovirus multiplication in these cells. Thus a relatively simple chemically defined medium is available for the study of the relationship between cell nutrition and poliovirus action. The effect of the withdrawal of specific metabolites from this maintenance medium on poliovirus action is the subject of this paper.

Materials and methods. Monkey kidney cell cultures. Trypsinized cells were grown in medium D(3) for 6 days either in test tubes or in 60-mm diameter Petri plates. After removal of the growth medium, the established cultures were washed with phosphate-buffered saline (PBS)(4): the tubes 3 times, each time with 2 ml, and the plates 3 times, each time with 4.25 ml. These cells were then maintained either on Eagle's medium for HeLa cells without serum, here referred to as Eagle's maintenance (E_m), or on E_m minus one or more of its component compounds. *Poliovirus strains.* Plaque-purified pools of 3 strains have been used. These are Akron purified pool #1 (PPl), Brooks PPl, and Mabie PPla, which are examples of antigenic types I, II, and III, respectively. These strains have been described previously (5,6,7). When not in use, the pools are stored in a dry-ice box. *Nutritional tests in tube cultures.* To each tube culture of monkey kidney cells bathed by .90 ml E_m or deficient E_m at pH 7.2 to 7.3 is added .10 ml of a 1000-fold dilution in PBS of one of the poliovirus pools. This provides inocula of 740, 8500, and 300 plaque-producing particles (PPP) per tube for Akron PPl, Brooks PPl, and Mabie PPla, respectively. Control tubes receive .10 ml PBS. The tubes are rolled at 36°C and examined for cytopathogenic action on the second day after inoculation and daily thereafter usually through the eighth day after inoculation. *Nutritional tests using the plaque method.* The method of Dulbecco and Vogt(4) as adapted for use in this laboratory(7) has been used with the modification that the plaques develop under E_m or deficient E_m agar in an incubator continuously flushed with 97% air and 3% carbon dioxide(4). *Sources of compounds tested.* All compounds were purchased from commercial firms except thiomalic acid and 2-mercaptoethylamine hydrochloride of which free samples were kindly supplied by Evans Chemetics, Inc., New York City. None of the compounds was further purified.

Results. Delay in appearance of cytopathogenic effect on monkey kidney cells in tube cultures in absence of cystine. The cytopathogenic action of Akron, Brooks, and Mabie is delayed when cystine is not supplied for the monkey kidney cell-poliovirus system (Table I). Similar experiments have shown that the omission of all 8 vitamins or of any one of the amino acids tyrosine, leucine, methionine, arginine, histidine, lysine, isoleucine, phenylalanine, threonine, tryptophane, and valine normally present in E_m does not result in any delay in the cytopathogenic action of Akron PPl. The omission of glutamine has given variable results, sometimes resulting in a short delay and at other times in no delay.

Retardation of plaque development in absence of cystine. The result, obtained in tube

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* Aided by a grant from the National Foundation for Infantile Paralysis.

TABLE I. Time of Appearance of Cytopathogenic Effect of 3 Strains of Poliovirus on Monkey Kidney Cells in Roller Tubes in Presence and in Absence of Exogenously Supplied L-cystine.

	Time required for completion of cytopathogenic action (mean day tubes positive)					
	1		2		3	
Poliovirus strain and pool*	Cystine†	No cystine‡	Cystine	No cystine	Cystine	No cystine
Akron PPl	3.0	$\geqq 6.8$	4.2	$\geqq 7.8$	3.0	$\geqq 8.0$
Brooks PPl	2.9	$\geqq 7.6$	3.2	$\geqq 7.5$	3.0	$\geqq 7.2$
Mabie PPla	2.9	$\geqq 7.2$	4.0	$\geqq 7.8$	3.0	$\geqq 7.8$

* Each virus pool was diluted 1000-fold in PBS before inoculation.
† Medium E_m with L-cystine at .20 mM.
‡ " " without L-cystine.

cultures, that exogenously supplied cystine is necessary for the normal action of the Akron, Brooks, and Mabie viruses on monkey kidney cells has been confirmed using the plaque technic (Table II).

Delay in poliovirus propagation in absence of cystine. Curves which show the propagation of Akron PPl on monkey kidney tissue cultures in the presence and in the absence of cystine were obtained by plaque assays of daily harvests of supernatant fluids from appropriate cultures. These curves show that the propagation of Akron is delayed in the absence of cystine to about the same extent as is the cytopathogenic effect of Akron. This slower propagation in the absence of cystine probably is not due to any interference with virus adsorption (see below). It may be due either to a decreased rate of virus multiplication or virus release or both.

TABLE II. Plaques Formed by Akron PPl, Brooks PPl, and Mabie PPla on Monkey Kidney Cells in Presence and in Absence of Exogenously Supplied L-cystine.

		Plaques/plate	
Poliovirus strain and pool	Virus conc.	E_m*	E_m without L-cystine
Akron PPl	10^{-3}	—	1
	10^{-4}	†	0, 0
	10^{-5}	20, 25	0
	$10^{-5.4}$	11	—
Brooks PPl	10^{-4}	—	0
	10^{-5}	63, 28	0, 0
	10^{-6}	3, 4	0, 0
Mabie PPla	10^{-4}	46, 46	5, 1
	10^{-5}	8, 4	1

* Contains L-cystine at .05 mM.
† Too many to count.
— = Not done.

The plaques developed for 4 days at about 35°C. In addition to the differences in assay found above, the plaques that were formed on E_m without cystine were smaller than those formed on E_m.

TABLE III. Amount of L-cystine Necessary for Normal Cytopathogenic Action by Akron PPl on Monkey Kidney Cells.

Conc. (mM) of L-cystine in maintenance medium	Time required for completion of cytopathogenic action (mean day tubes positive)
0	$\geqq 9.1$
.001	$\geqq 9.4$
.004	$\geqq 9.9$
.016	6.6
.064	4.3
.256	3.7

Each tube received .10 ml Akron PPl at 10^{-3} conc. in PBS.

Concentration of cystine necessary for optimal cytopathogenic action by Akron PP1. In Table III are presented data which show that the lowest concentration of L-cystine which will give optimal or near-optimal cytopathogenic action is about 10^{-4} M. The amount of cystine or "cystine-activity" in the inoculum itself has been estimated by the assay of autoclaved (10 lbs/in^2 for 10 minutes) samples of Akron PPl, Brooks PPl, and Mabie PPla, using the poliovirus-monkey kidney tissue culture system. In this way it was found that the amount of "cystine-activity" introduced into the system by the usual inoculum of 10^{-3} virus concentration is far too small to affect significantly the determination of the concentration of cystine necessary for optimal cytopathogenic effect.

Tests of compounds as possible substitutes for cystine. Seventeen compounds were selected on the basis of their metabolic relationship with cystine or simply because they possessed a chemical structure somewhat similar to that of cystine. These compounds were

TABLE IV. Activity of 17 Compounds in Replacing Cystine for Hastening Cytopathogenic Action of Akron PPl on Monkey Kidney Cells.

Activity relative to L-cystine		
Approximately equally active	Less active on a molar basis	Inactive‡
L-cysteine hydrochloride	DL-cystathionine*	DL-α-alanine
Glutathione	DL-homocysteine thiolactone hydrochloride†	Cysteic acid
		Glycine
		DL-homocystine (toxic; .1 mM)
		2-mercaptoacetic acid
		2-mercaptoethanol (toxic; .5 mM)
		2-mercaptoethylamine hydrochloride (toxic; .05 mM)
		DL-methionine§
		DL-serine
		Sodium sulfate
		" sulfite
		" thiosulfate
		Thiomalic acid

* About 8 times less active.

† " 20 " " " .

‡ Inactive at conc. of .10 and 1.0 mM except for the 3 compounds which were noticeably toxic for monkey kidney tissue cultures as designated above. Following the notation of toxicity is the highest relatively nontoxic conc. tested and found inactive.

§ Normally present in E_m at .05 mM.

tested for the ability to substitute for cystine in the latter's capacity to hasten the cytopathogenic action of Akron PPl. The results obtained permit the grouping of the compounds into 3 categories (Table IV). The possibility that the relatively low activities of DL-cystathionine and of DL-homocysteine thiolactone hydrochloride may be due to small amounts of contaminating substances, *e.g.*, cystine, has not been excluded.

Elimination of adsorption as the step at which cystine is effective. Particles of Akron PPl were adsorbed for 30 minutes at 36°C onto monolayers of monkey kidney cells, which had been washed 3 times with PBS (4.25 ml/washing), in the presence of 3 different media: PBS, E_m without cystine, and E_m with L-cystine at .50 mM. The fraction of the particles adsorbed was the same in the 3 media. This indicates that the presence of exogenously supplied cystine is effective at some postadsorptive phase(s) of the viral infection.

Discussion. Davies *et al.*(8) studied the effect of dietary amino acid deficiencies in mice on the course of poliomyelitis following intracerebral inoculation of a mouse-adapted strain of Lansing poliovirus. They found that a deficiency in any one of 8 amino acids (tryptophane, isoleucine, valine, phenylalanine, histidine, threonine, methionine, and leucine) resulted in some delay in the onset of symptoms, in a decrease in the total number of mice paralyzed by the 28th day postinoculation, and in an increase in the number of mice which died without showing characteristic signs of poliomyelitis. A lysine deficiency, however, had very little or no effect on the course of the disease. Their deficient mice and one of their control nondeficient groups received no cystine, cysteine, or glutathione. Their other control group received only the small amount of cystine present as residues in the casein fed. It is difficult to relate this work to the studies performed *in vitro* because of the manifold differences in system and technic. Recently Eagle and Habel have reported that salts, glucose, and glutamine are sufficient and necessary for poliovirus propagation on HeLa cells. A comparison of their findings with the results herein reported suggests that the cystine and possibly the glutamine requirements for poliovirus action depend on the nature of the host cell and/or the nature of the poliovirus strain. Further investigations, however, are needed to clarify the situation. Rappaport(9) has developed a medium containing salts, glucose, cysteine, and six other amino acids for the maintenance of monkey kidney tissue cultures during poliovirus propagation. Furthermore, she has found that cysteine can replace the 6 other amino acids for poliovirus propagation. The combined evidence strongly indicates that cysteine and/or its disulfide derivative cystine play an important role in the process of poliovirus propagation in monkey kidney tissue cultures. These results suggest the possibility of finding cystine antimetabolites which would delay poliovirus action *in vitro* and perhaps *in vivo*. With only the information available at this time it is somewhat premature to speculate as to the reason for this cystine requirement. One reasonable working hypothesis is that the available endogenous cystine, either free or in peptide bondage, simply does not meet the demand for the cystine residues of the protein portions of the newly-forming poliovirus particles.

Summary. The cytopathogenic action of the poliovirus strains Akron, Brooks, and Mabie on either tube or Petri plate cultures of monkey kidney cells is considerably delayed when cystine is omitted from the maintenance medium. Omission of all the vitamins or of any one of the 12 other amino acids except glutamine normally present in the maintenance medium resulted in no delay in the appearance of the cytopathogenic effect of Akron PPl. Omission of glutamine resulted in a short delay or no delay. It has further been shown that the propagation of Akron is slower in the absence of cystine. The lowest concentration of L-cystine which will give optimal or near-optimal cytopathogenic action by Akron is about 10^{-4} M. Glutathione and L-cysteine hydrochloride can replace cystine at about the same concentration; DL-homocysteine thiolactone hydrochloride and DL-cystathionine can replace also but only at higher molar concentrations. None of 13 other compounds was found to replace cystine. The adsorption of Akron onto monkey kidney cells is not affected by the absence of cystine.

1. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.
2. Eagle, H., *Science*, 1955, v122, 501.
3. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.
4. Dulbecco, R., and Vogt, M., *J. Exp. Med.*, 1954, v99, 167.
5. Wenner, H. A., and Kamitsuka, P., *J. Immunol.*, 1955, v75, 356.
6. Wenner, H. A., Kamitsuka, P., and Lenahan, M., with collaboration of Melnick, J. L., *ibid.*, 1956, in press.
7. Dubes, G. R., *Virology*, 1956, v2, 284.
8. Davies, W. L., Pond, W. L., Smith, S. C., Rasmussen, A. F., Jr., Elvehjem, C. A., and Clark, P. F., *J. Bact.*, 1952, v64, 571.
9. Rappaport, C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 464.

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Role of the Adrenal in Response of Rats to *Orinase*.* (22686)

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Adrenocortical and medullary hormones both counteract the hypoglycemic effects of insulin(1,2,3). Excessively high doses of the hypoglycemic agent *Orinase* do not produce a hypoglycemic response(4).[†] These observations suggested that the adrenals may respond to high doses of *Orinase* with increased secretions. Therefore, experiments were designed to study the role of the adrenal cortex and medulla in modifying the hypoglycemic reaction of rats to this drug.

Methods. Male rats obtained from the Upjohn colony (Sprague-Dawley ancestry) were used throughout these experiments. The intact animals weighed 140-160 g at the time of treatment with *Orinase*. Adrenalectomies and

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[*] Upjohn tradename for 1-butyl-3β-tolylsulfonylurea.

[†] Personal communication from W. L. Miller.

adrenal demedullations were performed at an average body weight of 150 g. Adrenalectomized rats were maintained on 1% NaCl as drinking fluid and were given the hypoglycemic drug on the 7th day postoperatively except in one experiment. In this case, as shown in Table II, it was administered on the 9th day after adrenalectomy. The O day represents the day of *Orinase*. The 500 μg/day dose of hydrocortisone was started on the 4th day postoperatively and given for 5 days, with the last injection given immediately prior to the *Orinase*. The 5 mg dose of hydrocortisone was given as one injection immediately prior to the sulfonylurea on day O. All hydrocortisone injections were given in a volume of 0.2 cc of CMC vehicle(5). When epinephrine[‡] was used it was given subcutaneously 1 hour following the oral administration of the hypoglycemic agent. The adrenal demedullated rats were kept at least 4 weeks before *Orinase* treatment in order to insure regeneration of the adrenal cortices. The functional activity of the adrenal cortices has been indicated[§] by the fact that these animals can withstand stress induced by intraperitoneal egg albumin and the glands weighed between 40-50 mg after 4 weeks. These rats were maintained on 1% sodium chloride as drinking fluid for the first week and then on tap water. *Orinase* was given orally in all cases. Doses up to 275 mg/kg were given in 0.5 cc of CMC vehicle(5) and higher doses in a volume of 2 cc. All animals were fasted 24 hours prior to *Orinase* administration. Blood samples were obtained from the vena cava 2½-3 hours after *Orinase* treatment while the animals were under sodium cyclopal anesthesia. The blood was oxalated and glucose was estimated by the Folin-Wu method(6). Liver glycogen was determined by the anthrone method on tissues removed 7 hours following treatment.

Results. The minimal effective hypoglycemic dose of *Orinase* in intact rats was approximately 100 mg/kg (Fig. 1). These data also show that the maximal fall in blood sugars was 36% at a dosage of 266 mg/kg. At the high dose of 2 g/kg there was no demonstrable depression of blood sugar in the intact rats. The same type of response was manifested in the deposition of liver glycogen under the influence of *Orinase* (Table I). While 266 mg/kg of the sulfonylurea produced a marked increase in liver glycogen, the higher doses caused less deposition.

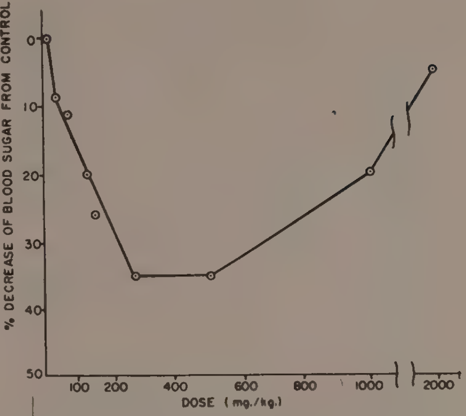

FIG. 1. Dose-response of intact rats to the hypoglycemic effects of *Orinase*. Each point represents 8 controls and 8 treated animals. Animals were bled 2½-3 hr following treatment.

In adrenalectomized rats as little as 9 mg/kg of *Orinase* caused a fall in blood sugar (Fig. 2). At dosages of 45 and 90 mg/kg the adrenalectomized rats were subject to convulsions and blood sugar levels were depressed by 45 and 57% from the controls. To establish that the convulsions were due to hypoglycemia an experiment was performed in which *Orinase* at a dosage of 100 mg/kg was administered orally to 20 adrenalectomized rats. One-half of these animals received 1 g of glucose orally after they were in

TABLE I. Effects of Varying Doses of *Orinase* on Fasting Liver* Glycogen.

Treatment	Dose	No.	Liver glycogen
Vehicle	2 cc	7	.24
Orinase	266 mg/kg	6	.66
	523	6	.57
	1048	6	.35
	2000	6	.34

* Livers were removed 7 hr following treatment.

[‡] Upjohn Epinephrine Hydrochloride 1:1000.

[§] Unpublished data.

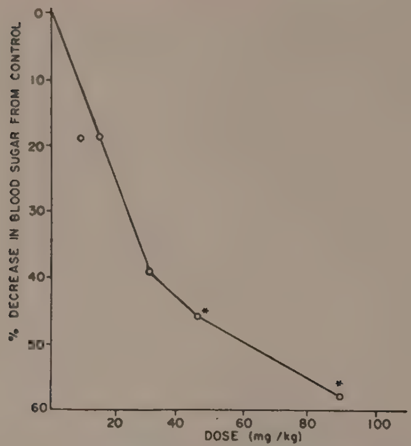

FIG. 2. Dose-response of adrenalectomized rats to *Orinase*. Each point represents 8 controls and 8 treated rats. Animals were bled 2½-3 hr following treatment.

* All animals were convulsing.

TABLE II. Effects of Hydrocortisone on Response* of Adrenalectomized Rats† to *Orinase*.

No. rats	Treatment	Days	Dose	Blood sugar (mg %)
4	Subq. saline Oral CMC	5 0‡	.2 cc	91
4	Subq. saline Subq. F Oral CMC	5 0 0	.2 cc 5.0 mg .5 cc	103
4	Subq. F Oral CMC	5 0	.5 mg .5 cc	100
4	Subq. F Oral *Orinase*	5 0	.5 mg 66.0 mg/kg	58
5	Subq. saline Subq. F Oral *Orinase*	5 0 0	.2 cc 5.0 mg 66.0 mg/kg	53§
5	Subq. saline Oral *Orinase*	5 0	.2 cc 66.0 mg/kg	38§

* Blood was taken 2½-3 hr following *Orinase* treatment.

† Avg body wt at time of *Orinase* was 160 g.

‡ 0 day was 9th day after adrenalectomy and at end of indicated treatment. The F 0-day and last inj. of F 5 days was given immediately prior to the *Orinase*.

§ Convulsing.

convulsions. In every instance the glucose stopped the convulsions and the animals were on their feet within 10 minutes. If glucose was not given, convulsions continued for several hours and usually ended in death.

In adrenal demedullated animals the minimal dose of *Orinase* causing hypoglycemia was between 15 and 30 mg/kg (Fig. 3). This is compared with 9-15 mg/kg for the hypoglycemic dose in adrenalectomized rats and 100 mg/kg for intact rats. Therefore, the adrenal demedullated animals are considerably more sensitive to the hypoglycemic effects of *Orinase* than the intact rat but do not seem to be quite as sensitive as the adrenalectomized animals. In addition, the dose of 2 g/kg produced essentially as much depression of blood sugar as the lower doses in the adrenal demedullated animals. It is important to note that the 2 g/kg dose was the maximum dose in this animal preparation since it killed approximately ½ of them.

To delineate further the role of the adrenal cortex and medulla in the response to *Orinase*, experiments were carried out to study the effects of hydrocortisone and epinephrine on blood sugar response of adrenalectomized rats to this drug. Treatment with hydrocortisone at 0.5 mg per rat per day for 5 days prior to *Orinase* administration in the adrenalectomized rats partially reversed the hypoglycemic effects of *Orinase* and increased the blood sugar somewhat over that of the controls (Table II). It is also interesting that a

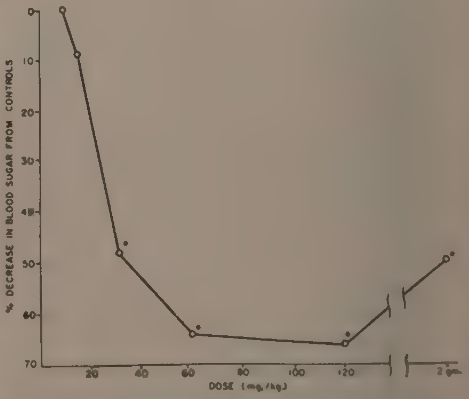

FIG. 3. Dose-response of adrenal demedullated rats to *Orinase*. Each point represents 8 controls and 8 treated animals except at the 2 g/kg dose. In this group there were 15 controls and 8 *Orinase*-treated as 7 treated rats died before they were bled. Animals were bled 2½-3 hr following treatment.

* All animals were convulsing.

TABLE III. Effect of *Orinase* plus Epinephrine on Blood* Sugar in Adrenalectomized Rats.

No. rats	Treatment	Dose	Blood sugar
5	CMC	0.5 cc	76
5	*Orinase*	66 mg/kg	40
7	*Orinase* +Epinephrine	66 " 200 μg/kg	116

* Blood was taken 2½-3 hr following the *Orinase*. Epinephrine was given 1 hr after the hypoglycemic drug.

single dose of 5 mg of hydrocortisone per rat given simultaneously with *Orinase* also partially reversed the extreme hypoglycemic effects with *Orinase* in this animal preparation. The blood sugar levels of the animals which received 5.0 mg of hydrocortisone were also somewhat higher than the untreated control adrenalectomized animals. The effect of epinephrine in reversing the hypoglycemic effect of *Orinase*, however, was more striking than that seen with hydrocortisone (Table III). When 200 μg/kg of epinephrine was administered to adrenalectomized rats 1 hour after oral administration of *Orinase*, the hypoglycemia was completely reversed and the blood sugar levels rose significantly above those of the untreated adrenalectomized controls.

Discussion. The difference in sensitivity of intact and adrenalectomized rats to *Orinase* shows that the adrenal is involved in the response of rats to this drug and agrees with previous observations(7) that adrenalectomized rats were more sensitive to the hypoglycemic effects of other sulfonamides than intact animals. The failure of high doses of *Orinase* to induce hypoglycemia or liver glycogen deposition in the intact rat may be the result of stimulation of epinephrine secretion by the adrenal medulla since epinephrine is known to increase blood sugar and deplete liver glycogen. The observations reported here which support this explanation are as follows: (1) the adrenal demedullated animals are more sensitive to the hypoglycemic effects of *Orinase* than the intact rats. (2) The hypoglycemic activity of 2 g/kg of *Orinase* was nearly as marked as that produced by lower doses in the demedullated rats. (3) Epinephrine completely reversed the hypoglycemic response of adrenalectomized rats to the sulfonylurea.

Since hydrocortisone partially reversed the hypoglycemic response of the adrenalectomized animal to *Orinase* and since adrenalectomized animals seem to be more sensitive to it than demedullated animals, it is probable that the adrenal cortex is also involved, but to a lesser degree. Another observation which suggested that the adrenal cortex may be stimulated with the high doses of *Orinase* was that there was slightly less hypoglycemic response at the 2 g/kg dose level as compared to that obtained with lower doses in adrenal demedullated rats.

These results also indicate that when intact animals are used to test hypoglycemic agents a wide range of dosages should be employed to be sure that the phenomenon of reversal of effects does not occur. Adrenalectomized rats are better test animals since they are extremely sensitive to hypoglycemic drugs and also the likelihood of masking the hypoglycemic effect by adrenal secretory activity induced by high doses is eliminated.

Summary. In the intact rat low doses of *Orinase* produced a significant depression of blood sugar and deposition of liver glycogen while higher doses were ineffective. The adrenalectomized rat was found to be approximately 10 times as sensitive and the adrenal demedullated rat 3-6 times more sensitive to *Orinase* than the intact rat. Hydrocortisone partially and epinephrine completely counteracted the hypoglycemic effects of *Orinase*. Failure of high doses of this sulfonylurea to produce hypoglycemia and liver glycogen deposition in the intact rats may be due to a stimulation of epinephrine secretion by the adrenal medulla. It is also suggested that glucocorticoid secretion is also stimulated by high doses of *Orinase* in the intact and adrenal demedullated animals.

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1. Selye, H., *Text Book of Endocrinology*, Acta Endocrinologica Inc., Montreal, Canada, 1949.

2. Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinol.*, 1940, v26, 309.

3. Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, Williams & Wilkins Co., Baltimore, Md., 1945.

4. Miller, W. L., and Dulin, W. E., *Science*, 1956, v123, 584.

5. Dulin, W. E., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 115.

6. Folin, O., and Wu, H., *J. B. C.*, 1920, v41, 367.

7. LaBarre, J., and Reuse, J., *Arch. Neerl. Physiol.*, 1947, v28, 475.

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Tubular Reabsorption of Protein in Experimentally Produced Proteinuria in Rats. (22687)

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It has long been recognized that cells lining the proximal convoluted tubule of the kidney of various animal species are capable of absorbing colloid substances from the tubule lumen(1-4); the process has been named *athrocytosis*. Dock(5) and Addis(6) were the first to suggest that under *normal* circumstances plasma proteins are to some extent filtered through the glomerulus of the kidney and reabsorbed by cells of the tubules. Because of the enormous volume of glomerular filtrate, a concentration of protein in the glomerular filtrate of only 15 mg/100 ml would result in excretion in the human of about 27 g of protein in 24 hours if the protein were not reabsorbed by the tubule cells. The work of many investigators has contributed to the concept of glomerular filtration and tubular reabsorption of plasma proteins in normal and abnormal conditions. These reports have recently been reviewed by Rather(7). Since this review, the quantitative aspects of tubular reabsorption of protein in the normal rat has been studied by Sellers, *et al.*(8) and the mechanism of tubular handling of reabsorbed protein has been investigated by Oliver and co-workers(9). Theoretically, a decrease in tubular reabsorption of plasma proteins filtered through normal glomerular basement membrane could account for the protein found in urine in most instances of proteinuria. Actually, however, in glomerulonephritis there is evidence of glomerular damage, and even in cases of idiopathic nephrotic syndrome, where only basement membrane thickening is found to suggest changes in glomerular permeability, calculations based on clearance studies in patients with massive proteinuria lead to the conclusion that glomerular permeability to plasma proteins is increased (10). This still leaves open the question of whether proteinuria occurs solely because filtration through abnormally permeable glomeruli exceeds the normal ability of tubules to reabsorb protein, or whether a decrease in tubular reabsorption of protein is an added factor in production of proteinuria.

The dye T-1824 (Evans Blue) binds firmly to serum proteins and has been used to measure renal clearance of albumin in humans(11) as well as tubular reabsorption of protein in rats(5,8,12). The amount of dye accumulating in droplet form in cells of the proximal convoluted tubule is assumed to be a measure of reabsorption from the tubule lumen of dye-labeled serum proteins. In the present report, dye-labeling of serum proteins has been used to study the role of tubular reabsorption of protein in 2 types of experimentally produced proteinuria.

Materials and methods. Female Osborne-Mendel rats of about 150 g body weight were used. Proteinuria due to uranium administration was produced by intravenous administration of 0.174 mg uranyl acetate containing 0.1 mg uranium, in 1.0 ml of 0.15 M saline. Proteinuria due to anti-kidney serum was induced by a single intravenous injection of rabbit anti-rat kidney serum. The nephrotoxic serum was obtained from rabbits immunized by repeated intraperitoneal injections of glomerular fraction of perfused rat kidney homogenate. To obtain urine for pro-

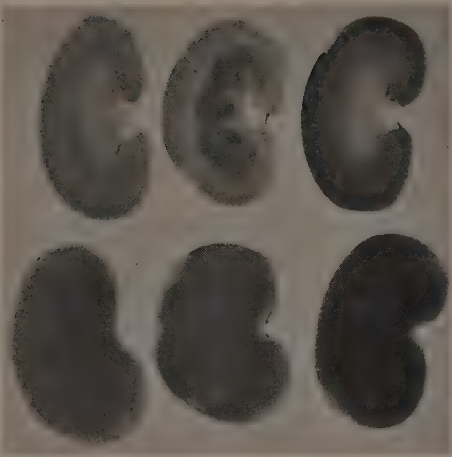

FIG. 1. Sagittal section and surface view of unfixed kidneys from control rat (left), rat with proteinuria produced by uranium inj. (center), and rat with proteinuria produced by inj. of nephrotoxic serum (right). Each rat received 2.5 mg T-1824 intrav. 24 hr before kidneys were removed.

tein determinations, rats were placed in individual metabolism cages with an adequate liquid diet containing casein hydrolysate in place of protein and urine was collected overnight. Proteinuria was measured by the method of Shevsky and Stafford(13). Two and one-half mg of T-1824 dye in 0.15 M saline were injected intravenously into a foot vein of rats followed by an overnight urine collection. Twenty-four hours after dye injection, the rats were anesthetized with ether, exsanguinated from the abdominal aorta, and both kidneys were decapsulated and removed. One kidney was sliced in half with a razor blade, placed in a Petri dish containing ice cold 0.15 M saline and examined in the gross and at various magnifications under a Leitz binocular dissecting microscope. The other kidney was fixed in 10% formalin and both frozen sections and hematoxylin and eosin and PAS stained sections were made. In another experiment, some animals were autopsied at daily intervals after antiserum injection and the kidneys were examined and weighed.

Results. Normal rats. After intravenous injection of 2.5 mg of T-1824 in normal rats, a normal straw or amber colored urine was obtained. Study under dissecting microscope of kidneys removed 24 hours after dye injection showed a pale green-tan or blue-green surface, and at higher magnifications none of the mosaic pattern of convoluted tubules was strikingly stained by the dye. On cut section (Fig. 1), the outer zone of the cortex was a faint but definite green, the inner zone was colorless and the medula was red. It was evident that relatively small amounts of the blue dye were present in the tubular cells.

Uranium proteinuria. It was observed in preliminary experiments, data from one of which are shown in Table I, that proteinuria was produced after a latent period of 2 or 3 days by 0.174 mg of uranyl acetate. This dose was chosen for 4 T-1824 experiments in 8 rats each. Forty-eight hours following injection of uranyl acetate, T-1824 dye was injected. Urine collected overnight was blue and contained protein. Twenty-four hours after T-1824 injection, the external surface of kidneys were a pale green color not unlike the T-1824 injected normal rat kidneys (Fig. 1), but at higher magnification the mosaic of tubules consisted of pale green colored tubules alternating with completely colorless tubules not observed in control rats. The cut section of kidneys showed a pale green outer cortex, containing somewhat less blue color than the T-1824 injected control kidneys, a colorless inner zone of cortex and the red medullary zone. Microscopic examination showed apparently normal glomeruli and changes in cells of proximal convoluted tubules consisting of marked necrosis and even desquamation of cells from the tubular basement membranes.

Nephrotoxic serum proteinuria. After intravenous injection of a standard dose of rabbit anti-rat kidney serum (twice the minimum amount required to produce proteinuria), the 150 g female rats developed immediate proteinuria. In most rats proteinuria persisted indefinitely, but in some rats it gradually subsided. Definite renal enlargement occurred in 2 or 3 days. T-1824 studies were made as early as 24 hours and at various longer periods after antiserum injection in 17 rats with chronic proteinuria due to nephrotoxic serum. The urine collected after T-1824 injection contained about 7 mg/hr of protein and was blue. When examined 24 hours after T-1824

TABLE I. Time of Onset of the Proteinuria Produced by Intravenous Injection of Uranium and Nephrotoxic Serum.

		Proteinuria				
		Day 1	Day 2	Day 3	Day 4	Day 7
Material inj.	No. rats	mg/hr				
Saline (controls)	4	.3	.2	.1	.2	.2
.0178 mg uranyl acetate	2	.2	.1	.3	.3	.2
.178 mg " "	8	.3	.7	1.6	1.0	.5
1.78 mg " "	4	1.1	3.5*			
Nephrotoxic serum	4	11.0	5.1	4.0*		

* Sacrificed for autopsy examination.

injection the surfaces of kidneys were deep blue and under higher magnification one could see against the background of blue-stained tubules an occasional convoluted tubule which was an even more intense blue (Fig. 1). At highest magnification the blue could be seen to be in granular form, apparently in the tubule cells. The cut sections showed intense blue staining of outer and inner zones of the cortex; the medulla was red. Frozen sections of such kidneys after formalin fixation showed the blue granules or droplets in the cytoplasm of the cells lining the proximal convoluted tubules as has been described by other investigators in kidneys of normal rats 24 hours after intravenous injection of much larger amounts (25 mg) of T-1824(8,12). Varying degrees of basement membrane thickening and patchy lipid deposition in tubule cells were present.

It should be noted that the amount of T-1824 injected in our experiments was sufficiently small that it should have been completely bound to albumin even in chronically proteinuric rats with lowered plasma albumin levels. To avoid complication of the difference in plasma albumin levels between control and nephrotic rats, 2.5 mg of T-1824 were administered to 12 normal rats and followed within a few minutes in 6 rats by nephrotoxic antiserum. In those rats receiving nephrotoxic serum, there was deep blue staining of the cortex in 24 hours as described in the chronically proteinuric rats.

Discussion. Previous investigators have summarized the facts and the reasoning behind the assumption that the appearance of the T-1824 dye in the cytoplasm of proximal convoluted tubule cells represents dye-labeled protein which has been filtered through the glomerulus and reabsorbed from the tubule lumen by proximal convoluted tubule cells(5, 8,12). The T-1824 in serum and urine of proteinuric rats was all precipitated with the protein by protein precipitating agents in the experiments of Gilson(12), and Sellers, *et al.* (14), and in our own experiments.

In the present experiments, the injection of one-tenth of the amounts of T-1824 used in experiments of others with normal rats(12, 14) resulted in appearance of only a small amount of color in the cortex of normal rats after 24 hours. In rats with proteinuria resulting from anti-kidney serum administration, appearance of large amounts of T-1824 in the tubule cells probably indicates that dye-labeled protein entered the cells in larger amounts than in the T-1824 injected control rats. This is consistent with the hypothesis that an increase in glomerular permeability due to antiserum damage led to increased filtration of dye-labeled serum proteins through the glomerulus, followed by increased tubular reabsorption of dye-labeled protein. The greater than normal accumulation of blue dye in 24 hours in the tubule cells of rats which received T-1824 prior to nephrotoxic serum administration seems to us to eliminate the possibility that the accumulation of dye in nephrotic rats is due to the presence of a larger amount of unbound dye in nephrotic rats than in controls. An alternative explanation for our finding is that damage to the tubule cells interfered with the mechanisms for disposing of the reabsorbed dye so that an increase in the dye in tubules occurred without an increase in tubular reabsorption of protein. Consideration of the findings in the kidneys of rats with uranium damaged tubules makes this possibility seem unlikely.

The renal enlargement which occurs in rats receiving anti-kidney serum presumably is related to the increased reabsorption and perhaps degradation of filtered protein; this is suggested by the observation of similar enlargement when proteinuria was produced by repeated infusions of protein in normal rats (15).

The rats injected with 0.1 mg uranium intravenously developed proteinuria only after 48 to 72 hours. Uranium is transported in combination with serum albumin and bicarbonate(16). Since almost all the injected uranium leaves the blood stream, and concentration in the kidney is greatest in the first few hours(17), it is difficult to account for the delay in onset of proteinuria. Tubular reabsorption of uranium from the tubule lumen would account for the well known localization of uranium produced renal damage in the proximal convoluted tubule(17). It is also consistent with the concept that the proteinuria produced in uranium injected rats is due primarily to decreased tubular reabsorption of protein by damaged tubule cells since no increase, and in fact a probable decrease, in T-1824 accumulation by tubule cells is found. Since no glomerular damage is detected in the presence of obvious localized tubular necrosis in such rats, it is tempting to consider that the protein appearing in urine is normally filtered plasma protein which is no longer reabsorbed by damaged tubule cells. One cannot, however, rule out the possibility that an increase in glomerular permeability has occurred in addition to decreased tubular reabsorption of protein.

The findings indicative of increased reabsorption of dye-labeled protein by the tubule cells of the kidneys of rats injected with antirat kidney serum are in accord with similar observations made by Spector(18) using radioactive iodine as the tag for serum protein. It should be noted that the rats receiving antikidney serum develop hypoproteinemia and lipemia and have renal lesions which are similar to those observed in many cases of the nephrotic syndrome in man(19).

Summary. An attempt was made by labeling plasma proteins with the blue dye T-1824 to estimate the amount of protein filtered and reabsorbed by the kidney under several conditions. A single intravenous injection of 2.5 mg of T-1824 into normal rats did not cause the urine to be blue and the kidneys examined 24 hours after the injection exhibited only faint blue staining of the proximal convoluted tubules of the cortex. When proteinuria was produced by a single small injection of uranyl acetate, the injection of T-1824 was followed by blue staining of the urine. However, the cortices of the kidneys contained less blue dye than those of control animals, indicating, presumably, that there was less protein reabsorption than normal by the damaged tubular cells. Injection of the blue dye into nephrotic rats with proteinuria due to administration of nephrotoxic anti-rat kidney serum was followed by blue staining of the urine and intense blue staining of the cortices of the kidneys. This presumably indicated that protein reabsorption by the tubular cells was greater than normal, and that the proteinuria was a result of increased glomerular permeability and not of decreased tubular reabsorption of normally filtered protein.

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1. Suzuki, T., *Zur Morphologie der Nierenskretion,* 1912, Jena, Fischer.

2. Gerard, P., *Comp. Rend. Soc. Biol.,* 1933, v115, 199.

3. Lambert, P., *ibid.,* 1933, v114, 1370.

4. Smetana, H., and Johnson, F. R., *Am. J. Path.,* 1942, v109, 1029.

5. Dock, W., *N. Eng. J. Med.,* 1952, v227, 663.

6. Addis, T., *Tr. Assn. Am. Phys.,* 1942, v57, 106.

7. Rather, L., *Medicine,* 1952, v31, 357.

8. Sellers, A. L., Griggs, N., Marmorston, J., and Goodman, H. C., *J. Exp. Med.,* 1954, v100, 1.

9. Oliver, J., MacDowell, M., Lee, Y. C., Moses, M. J., Kretchmer, N., Dickerman, H. W., and Cherot, F., *J. Exp. Med.,* 1954, v99, 589.

10. Chinard, F. P., Lauson, H. D., Eder, H. A., Greif, R. L., and Hiller, A., *J. Clin. Inv.,* 1954, v33, 621.

11. Chinard, F. P., Lauson, H. D., and Eder, H. A., *ibid.,* 1952, vv31, 895.

12. Gilson, S. B., Proc. Soc. Exp. Biol. and Med., 1949, v72, 608.

13. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry; Methods,* Baltimore, 1932, 682.

14. Sellers, A. L., Smith, S., Marmorston, J., and Goodman, H. C., *J. Exp. Med.,* 1952, v96, 643.

15. Baxter, J. H., and Cotzias, G. C., *J. Exp. Med.,*

1949, v89, 643.

16. Barron, E. S. G., *Toxicology of Uranium, National Nuclear Energy Series,* 1951, vIV-23, p41, McGraw Hill, New York.

17. Newman, W. F., *Pharmacology and Toxicology of Uranium Compounds, National Nuclear Energy Series,* 1951, vVI-1, p707, McGraw Hill, New York.

18. Spector, W. G., *J. Path. and Bact.*, 1954, v68, 187.

19. Heymann, W., and Lund, H. Z., *Pediatrics,* 1951, v7, 691.

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Genic Control and Hormonal Reversal of Sex Differentiation in Xenopus.* (22688)

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It has long been realized that in the cytodifferentiation of germ cells not only genes and external environmental factors play important roles, but also certain conditions of their immediate milieu(1). The first figure presents diagrammatically the basic facts and their interpretation based on recent work. Germ cells, on arriving in the primitive gonadal cortex, become invested each by a *nurse or follicle cell.* If unopposed, the follicle eventually induces development of the germ cell into an egg. However, if at the critical stage of sex differentiation a germ cell becomes located in the medulla, another inductor system, the *interstitial cells,* upsets the influence of the follicle cells and leads to the spermatogenetic type of differentiation.

How can such an interpretation be integrated with the well known facts of genic control of sex ratios? The present experiments seem to shed some new light on this problem.

Material and methods. Xenopus laevis Daudin has been bred in our laboratory since 1949 without addition of new stock. The experimental larvae were placed in hormone solutions of given concentrations, the solutions being completely replaced every 24 hours. The temperature was kept constant at 20°C.

Experiments. Since in the adult organism follicles and interstitial cells produce estrogenic and androgenic steroids, it was necessary to investigate the possibility that in embryonic sex differentiation the same hormones might first play the role of inductor substances. Experiments with Xenopus gave very interesting results, although not in the sense of a confirmation of the tentative assumption. Small or large doses of androgens added to the aquarium water equally produce what first seemed to be *all premature male cultures.* Given throughout the larval period they yield at metamorphosis little toads that all have well developed dark arm and hand pads and immediately start embracing each other. However, on dissection it is found that only about half of the animals have testes, the other half have normal ovaries (Fig. 2). We shall later come back to this experiment but next must describe the effect of the opposite treatment: exposing the larvae to estrogenic hormones, usually *estradiol.* Here the entire offspring is completely feminized. The original experiments with Allison(2,3) have often been repeated and thousands of offspring have been produced that consisted of females only(4,5). Some recent data are presented in Fig. 3.

These two hormone effects are all the more remarkable, because they are in contrast with the results of parabiosis and gonad transplantation, as described by Chang(6). The simultaneous presence of testes and ovaries in a system with a common blood circulation always results in normal testes and greatly suppressed ovaries. Sometimes even testicu-

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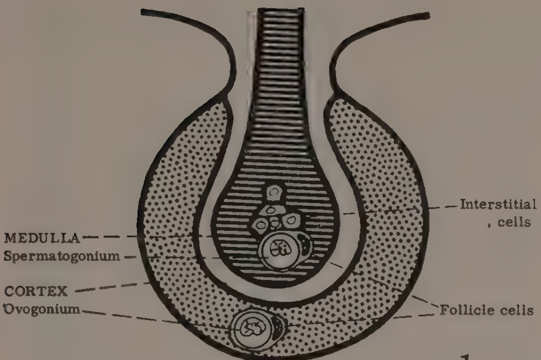
* Aided by a grant from the Rockefeller Foundation. The authors are also indebted to Ciba Pharmaceutical Products, Inc., for the generous supply of the steroid hormones.



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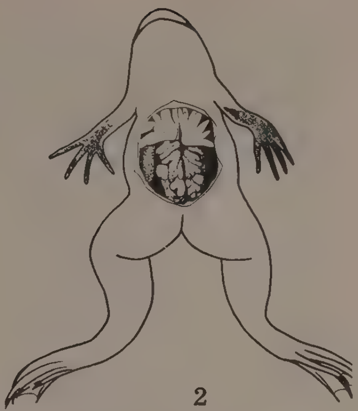

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FIG. 1. Diagram of cortical and medullary inductors of sex differentiation.
FIG. 2. Genetic female (ZW) which while under testosterone administrations developed ovaries and male secondary sex characters.

lar nodules appear in the latter.

The striking difference between these reactions proves that the inductor substances are not steroid hormones; for even if the male parabiont were producing an androgen, our first experiment shows that it could only stimulate secondary sex characters but not inhibit ovarial development. On the other hand, if the female partner were liberating small amounts of estrogens, the genetically male co-twin should develop ovaries—which again is not so. What then is the nature *of the inductor substance?* Further analysis of this problem must be preceded by a clarification of the role played by the *sex genes*(Fig. 3).

In Xenopus normally the female is heterozygous (ZW) and the male homozygous (ZZ). Therefore, treating part of the lay of a normal pair with estradiol should give an all female offspring in which half are of ZZ constitution. This had to be verified in breeding tests, for both ZZ and ZW animals are of the same appearance. Of 43 tested in this way, 22 gave an F_1 generation with the normal 1:1 ratio (ZW females): but 21 produced entirely male offspring and thereby proved to be of ZZ constitution. Evidently we can now dispense with the heterozygous sex type. Breeding homozygous stock only, one may produce any desired number of females by estradiol treatments at the larval stage. The possibility of controlling the sex ratio at the will of the experimenter is often a great convenience.

These breeding experiments with sex reversed toads spread some revealing light on the problem of the nature of sex determining genes. Since estradiol need only be administered during a short larval period, it is evident that the entire process of ovogenesis and the development of female secondary sex characters, including sex behavior, are no longer under the control of the sex genes. Apparently the genes play an active role only during a short and definite period, starting a chain reaction which later runs off independently. In trying to locate this period we first treated several 100% ZZ (male) cultures for 1-week periods with estradiol, and soon found that the critical time lies at the transition of stages 26 and 27(7), which actually is the time of initial embryonic differentiation (Table I). Attempts to pinpoint more exactly the time of interference with gene action led to surprisingly clear results. The primitive undifferentiated gonad of Xenopus consists of a series of about 15 pseudosegments or gonomeres. In accordance with general vertebrate development, they form and mature in craniocaudal sequence. If 2-day administrations of estradiol are given at the end of stage 26, the tops of the gonads differentiate into ovary, but the lower part remains unaffected and develops according to the testicular type (Fig. 4b, 5b). If, however, treatment is delayed until the

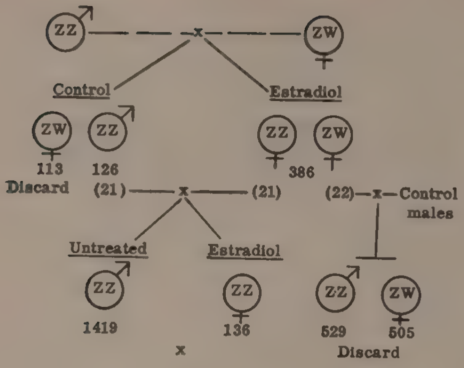


FIG. 3

FIG. 3. Experimental breeding record of Xenopus.

TABLE I. Series of All-Male (ZZ) Larvae, Each Treated during One of 4 Successive Weeks, with Estradiol, 50 $\mu g/l$.

Treatment	Stage (7)	N1*	N2†	♂	☿	♀
1st wk	24–25	47	38	38		
2nd	25–26	47	41	38	3	
3rd	26–27	72	65		4	61
4th	27–28	47	41	41		
Continuous	24–29	80	69			69
None		292	278	278		

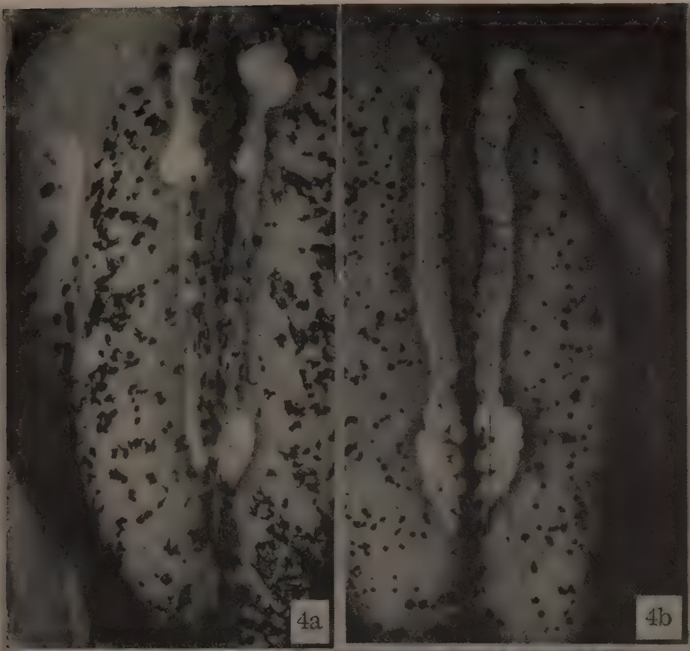
* Original No. of tadpoles.
† No. of tadpoles survived.

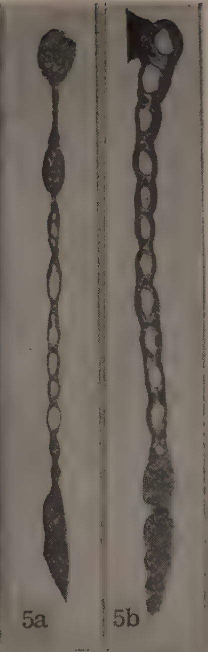
start of stage 27, the lower gonomeres become ovarial lobes. One may even produce triple sequences with ovary in the center and testis at both poles (Fig. 4a, 5a). In average cultures complete feminization requires close to 3 days. This means that about every 5 hours a new gonomere enters the responsive phase.

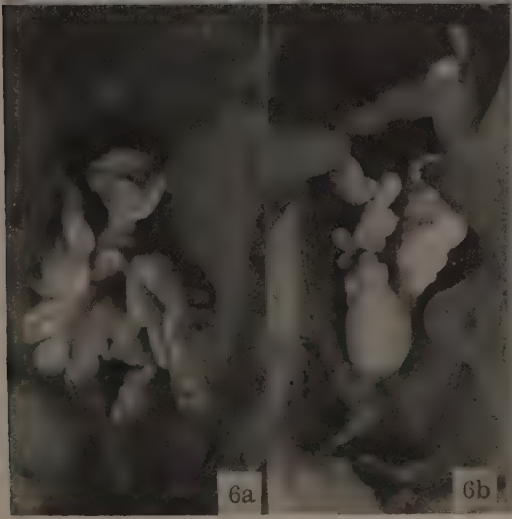
These experiments help in clarifying some aspects of the problem of *gene action*, recently discussed by Curt Stern(8). In general, the breeding of sex-reversed males in successive generations gives proof that the upset of genic expression neither induces, nor depends upon perceptible readjustments in the genic pool. This conclusion is unavoidable here, because the very cells that develop into eggs instead of sperms carry the genes into the subsequent generations. Also it becomes evident that the results of gene action are in time and quality co-determined by preadjusted factors of the internal milieu. In the present case the internal milieu is represented by inductor cells and substances. These are mostly proteins while the genes are DNA compounds. Delbrück has presented some interesting thoughts regarding the possible relationships between these widely different chemical classes(9). For a more penetrating discussion much more precise knowledge is needed especially regarding the inductor substances. In sex differentiation of gonochorists (*i.e.*, species with separate male and female sexes) there are at least two classes separately recognizable. Cortex and medulla each produces an agent, *corticin* and *medullarin* respectively, which induce ovogenesis or spermatogenesis. While in primitive hermaphrodite species the inductions by these substances may proceed side by side, even within one and the same gonad, in gonochorists the agents assume the character of antigens, so that the cortex now produces also an *anti-medullarin* and the medulla an *anti-corticin*. These "antibodies" have a wider range of action than the primary inductor substances and, as parabiosis experiments prove, they are proteins of high specificity that often are carried by the blood stream. These conclusions are partly a reinterpretation of previously reported experiments, but they are also based on the following new observations on Xenopus.

It was mentioned above that in male-female parabiosis, the ovary becomes extremely reduced. The same is the case if a testis is implanted into the body cavity of an early female larva. Interestingly, the same inhibition is not exerted by the testicular lobes of partially feminized males. Quite to the contrary, the ovarial lobes grow at about the same rate as ovaries of completely feminized larvae (Fig. 6). The full spermatogenic development in the testicular segment remains unaffected by the proximity of ovarial, also normally maturing lobes (Fig. 7). This is new evidence of the separate existence of the inductor of male differentiation (medullarin) and the inhibitor of ovarial differentiation (anti-corticin). Estradiol administration blocks the former only during actual treatment but the latter more generally and lastingly.

The rule that androgens have no sex-reversing effect on female larvae becomes







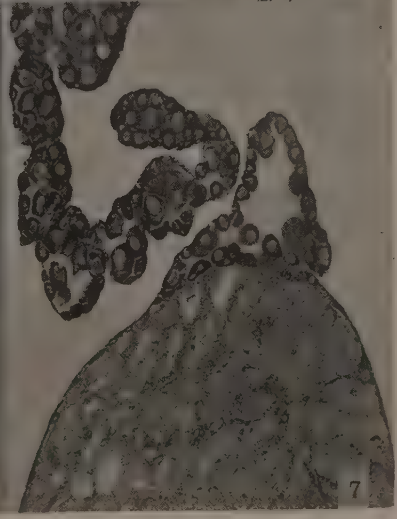


FIG. 4. Newly metamorphosed hermaphrodites of ZZ stock treated for 3 days with estradiol; a. ovarial gonomeres in the center of the sex glands; b. ovarial gonomeres above the (broader) testicular parts; × 19.

FIG. 5. Frontal sections through one sex gland each of 4a and 4b. Ovarial gonomeres with central cavities; ×27.

FIG. 6. ZZ males treated with estradiol, preserved 4½ mo. after metamorphosis; a. completely feminized; b. with a testicular segment in the lower right gonad; × 5.

FIG. 7. Longitudinal section through lower right gonad of 6b showing hermaphrodite mosaic; × 40.

TABLE II. Double Treatment with Estradiol, 50 $\mu g/l$, Followed by Testosterone, 50 $\mu g/l$, until Metamorphosis.

	Treatment	♂	☿	♀
A.	Control	55	—	—
	Estradiol (7 days)	—	1	17
	Estradiol followed by testosterone	16	5	—
B.	Control	72	—	63
	Estradiol (7 days)	1	5	18
	Estradiol followed by testosterone	7	4	10

slightly modified in the following experiment. Genetic males (ZZ), treated at the proper time with estradiol, become changed into females, if reared later in pure water. However, if the estradiol treatment is immediately followed by administration of testosterone, the animals revert back to males. Table II presents 2 experiments with identical procedures. But in the first one (A) the mother was a sex reversed male (ZZ) while in the second (B) she was of normal ZW constitution. Both experiments show that estradiol treatments feminize offspring of the ZZ type; but if it is immediately followed by testosterone, the reversion is wiped out, all ZZ individuals become males again (or intersexes). Under exactly the same conditions the ZW individuals develop normally into females. This abolition of initiated sex reversal is the only indication that androgens may inhibit the cortical inductors in a similar though much less effective way as estrogens are suppressing the medullary system.

Conclusions and summary. Continuation of experiments on sex determination, particularly with Xenopus, led to the conclusion that the primary inductors, corticin (of follicle cells) and medullarin (of interstitial cells) are antigens. The corresponding *anti-corticin* and *anti-medullarin*, suppressing the contrary inductor system, are responsible for the unisexual (gonochoristic) development of each individual. Normally the decision of cortical (female) or medullary (male) prevalence depends on the quantitative relationship between sex determining genes. Experimental reversal of this influence does not produce, or depend on, any perceptible temporary or permanent change in gene composition or function. If administered at a short critical period of sex determination (St. 26/27), estradiol causes ZZ larvae to develop permanently as females. This effect is brought about by depression of the medullary inductor system. The possibly ensuing development of hermaphrodite glands indicates that selectively the capacity of antibody (anticorticin) formation is more generally affected than that of the antigen (medullarin).

1. Witschi, E., *A. mikr. Anat.*, 1914, v86, 1.

2. Witschi, E., and Allison, J., *Anat. Rec.*, 1950, v108, 589.

3. Witschi, E., *A. Anat. micr. et Morph. exp.*, 1950, v39, 215.

4. Chang, C. Y., and Witschi, E., PROC. SOC. EXP BIOL. AND MED., 1955, v89, 150.

5. Gallien, L., *C. rend. Acad. Sci.*, 1953, v237, 1565.

6. Chang, C. Y., *J. Exp. Zool.*, 1953, v123, 1.

7. Witschi, E., *Development of Vetebrates,* Saunders, Philadelphia & London, (1956).

8. Stern, C., *Analysis of Development,* Saunders, Philadelphia and London, (1955), 151.

9. Delbrück, M., *Angew. Chem.*, 1954, v66, 391.

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Differential Distribution of Enzyme Hydrolyzing Acetylsalicylic Acid (Aspirin) in Certain Fluids of the Rat.* (22689)

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It appears likely that degradation of salicylic acid occurs principally, though probably not exclusively, in the kidneys since metabolites appearing in urine are not recoverable from the blood(1). The process is probably a complex one involving filtration at

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* Partially supported by a grant from the Wisconsin Heart Assn.

the glomerulus, tubular reabsorption, tubular conjugation and tubular excretion. However, the conversion of acetylsalicylic acid (hereinafter referred to simply as aspirin) into salicylic and acetic acids apparently takes place widely throughout the body(2), and it therefore becomes desirable to learn whether there is any differential distribution in various body fluids of the enzyme that catalyzes this hydrolysis. The object of the present study was to determine whether there are differences in rates of *in vitro* production of salicylic acid when aspirin is incubated in presumable immediate fluids of contact after gastrointestinal absorption in the rat (*i.e.*, abdominal thoracic duct lymph and portal vein blood) as contrasted with the systemically circulating blood.

Methods. Obtaining the fluids. Male Sprague-Dawley rats weighing between 175 and 225 g and full-fed on our usual adequate laboratory diet until the morning of the experiments, were used in 3 groups. In the group in which lymph was to be diverted for collection outside the body the animals were anesthetized with ether and the thoracic duct cannulated by the method of Bollman *et al.* (3). This technic comprises entrance into abdominal cavity through a large left flank incision, puncture of thoracic duct at approximately 1.5 cm above the receptaculum chyli, tying in a polyethylene tube (O.D. 0.062 cm, I.D. 0.045 cm) for drainage of lymph, and bringing the tube out of the cavity through puncture wound in midline of abdomen. On completing the silk-suture closure both the left flank wound site and that of the stab are painted over with collodion. The anesthetic is then withdrawn and lymph collection begins with animal confined in the Bollman cage(4), in which considerable freedom of movement is allowed without permitting claws or teeth to reach wound or protruding tube. Lymph was collected during 6 hours in a small centrifuge tube into which was placed at the beginning one small drop from a No. 27 needle of heparin sodium solution (10,000 units/ml) and a similar drop added as each ml of lymph accumulated. The collection tube was continuously twirled in beaker of ice water to insure that enzyme activity stopped at the moment a drop of lymph entered it and refrigerated at 4°C at end of sixth hour. In all instances, with 2 or 3 animals producing simultaneously, a lymph pool adequate for preparation of the aspirin solution was obtained through collections on 2 or 3 consecutive days. Animals bled from portal vein were anesthetized with ether, the abdomen opened in midline from manubrium sterni to symphysis pubis, the gastrointestinal structures displaced to gain access to the portal vein, the latter tied off close to point of entrance into the liver, and 2 ml of blood collected through syringe and needle, the latter containing 2 small drops of heparin solution, by direct puncture of the vein. The individual blood specimens were quickly cooled by twirling in beaker of ice water and a sufficient number accumulated to supply a plasma pool of equal size to that of lymph. The plasma obtained by centrifugation at 3500 r.p.m. for 10 minutes was stored in refrigerator. The blood specimens that were to represent systemically circulating blood were obtained by direct heart puncture under ether anesthesia of the third group of rats, cooling the 2 ml heparinized specimens as in the case of portal vein bloods and refrigerating the pooled plasma. *Determination of enzymatic hydrolysis.* On second or third day the 3 separate pools (lymph, portal vein plasma and heart's blood plasma) were filtered and 10 ml of each were placed in a separate flask into which aspirin had been previously introduced. The flasks were shaken vigorously for 2 minutes, then incubated at 38°C together with a flask containing only water and aspirin. The concentration of aspirin in all flasks was 50 mg/100 ml. At precisely 20, 40, 60, 80, 100 and 120 minutes after beginning of incubation, 1 cc aliquots were withdrawn from the flasks and the salicylic acid content determined by the method of Brodie *et al.*(5), in which the salicylic acid is extracted in acidulated ethylene dichloride, transferred to the water phase, an iron reagent added to give color, and read in the spectrophotometer at 540 millimicrons.

Results. The recoveries of salicylic acid from the 3 fluids are shown in Fig. 1, in which the data are charted as percentage recovered

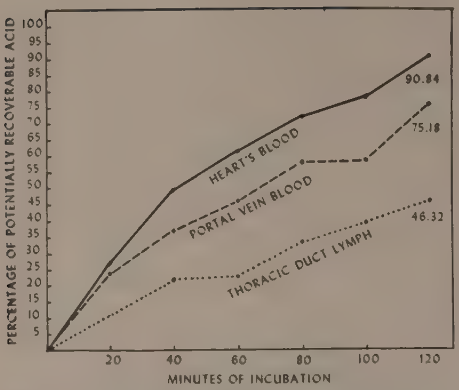

FIG. 1. Percentage recovered of potentially recoverable salicylic acid after *in vitro* incubation of acetylsalicylic acid in the respective fluids (rat).

of the potentially recoverable acid, using the means of 4 separate pools of each fluid at each of the time intervals. The highest recoveries at all intervals were from heart's blood, next highest from portal vein blood, and lowest from lymph. Throughout the entire 2-hour period the hydrolysis of aspirin proceeded at only about one-half the rate in lymph as in heart's blood. It is doubtful if the findings in the portal vein blood are truly representative of the enzymatic-hydrolysis-potential of that fluid directly as it leaves the site of principal aspirin absorption in the small intestine, because the specimens were taken without tying off the confluent veins from the stomach, spleen and pancreas, and therefore they contain some blood that had recently traversed the organs named. The presently designated portal vein blood is therefore actually such blood as it is delivered to the liver but it is not truly the first sanguine fluid that is encountered as aspirin is absorbed.

Discussion. The data establish that aspirin is hydrolyzed much less rapidly in lymph than in blood. It will be the objective of subsequent studies to determine whether the respective rates can be influenced by any adjuvant measures in the hope that the findings may help in the development of a satisfactory technic for determining salicylate antiphlogistic action in the laboratory.

Summary. The *in vitro* enzymatic hydrolysis of acetylsalicylic acid (aspirin) has been found to proceed only about one-half as fast in the abdominal thoracic duct lymph of the rat as in the heart's blood of the same animal.

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1. Gutman, A. B., Yü, T. F., and Sirota, J. H., *J. Clin. Invest.*, 1955, v34, 711; Hoffman, W. S., and Nobe, C., *J. Lab. and Clin. Med.*, 1950, v35, 237.

2. Smith, P. K., *J. Pharmacol. and Exp. Therap.*, 1949, v97, 353.

3. Bollman, J. L., Cain, J. C., and Grindlay, J. H., *J. Lab. and Clin. Med.*, 1948, v33, 1349.

4. Bollman, J. L., *ibid.*, 1948, v33, 1348.

5. Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharmacol. and Exp. Therap.*, 1944, v80, 114.

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Two Types of γ-Globulin Differing in Carbohydrate Content. (22690)

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Considerable evidence has accumulated suggesting that certain antibodies in human (1,2,3) as well as horse sera(4,5) are of higher molecular weight than other antibodies and the bulk of γ-globulin. A sedimentation constant of approximately 19 S has usually been observed in the ultracentrifuge indicating a molecular weight close to 1 million instead of the ordinary 150,000. The possibility has been raised that these large proteins are aggregates of the low molecular weight components(6). Recent experiments employing zone electrophoresis(7) have furnished evidence that normal human γ-globulin contains from 5-10% of 19 S material as a constant constituent. This is localized primarily in the faster migrating portion of the γ-globulin. Gamma globulin prepared by

Deutsch employing alcohol fractionation has been found to contain significant amounts of this heavy component(1).

Preliminary observations(7) indicated that this high molecular weight γ-globulin was very rich in carbohydrate as compared to the main 7 S fraction. The latter was found to contain approximately 2.5% of carbohydrate consisting of hexose sugars, hexosamine and sialic acid which remained quite constant in various preparations. The present report describes further observations on the considerably higher carbohydrate content of the 19 S component of γ-globulin which has been purified by repeated preparative ultracentrifugation.

Methods. Zone electrophoresis was carried out primarily in a polyvinyl chloride supporting medium(7) because this was particularly suitable for carbohydrate analyses of isolated fractions. Starch was also used as supporting medium but not in those instances where carbohydrate analyses were done. Both media were employed in a similar manner and the amount of 19 S γ-globulin isolated by the two methods was quite comparable. Several preparations highly enriched in heavy material were prepared from pooled normal serum by preparative ultracentrifugation in a Spinco Model E ultracentrifuge. In one instance 864 cc of normal human serum were recycled 8 times at 114,000 x G. The times chosen were sufficient to sediment the heavy material into the bottom of the tube, and depended on the total protein concentrations of the solutions. Beginning with the fifth centrifugation albumin was added in an attempt to prevent denaturation of the heavy material. The final preparation, consisting of primarily 19 S material and albumin, was separated by zone electrophoresis employing polyvinyl chloride as a supporting medium. Analytical ultracentrifugation of the fractions was carried out in a Spinco Model E ultracentrifuge and the composition was determined by methods described by Trautman (8). Hexose sugars were determined colorimetrically using the anthrone method according to Mokrasch(9). The coefficient of variation (V) was 3.2%. The color obtained in the anthrone-glycoprotein reaction was corrected for the color caused by protein-sulfuric acid interaction. The O.D. of an equally treated protein-sulfuric acid blank was subtracted from the O.D. measured after the anthrone reaction. The hexose sugars present in serum glycoproteins which are responsible for the anthrone color are mannose, galactose and 6-deoxygalactose. The latter sugar was separately assayed by employing the cystine method described by Dische and Shettles(10). V = 5.4%. For the determination of hexosamine the procedure described by Boas(11) was used chiefly. The proteins were hydrolyzed in 1.5 N HCl for 14 hours. Dowex 50 was used to eliminate non-specific chromogens and alkali treatment which is part of Ehrlich's reaction was carried out for 45 minutes at 90°C. V = 3.8%.

Sialic acid was assayed according to the method of Werner and Odin(12) which is based on Bial's orcinol reaction. Although pentoses and hexuronic acid produce color in this reaction the absorption maxima of their color products differ greatly from the maximal absorption of the color caused by sialic acid. The absorption curves of 19 S γ-globulin and 19 S α_2-globulin after the orcinol reaction are almost parallel to that of sialic acid, and their absorption maxima coincide with that of the latter at 570 mμ. No other maximum was observed which could indicate the presence of either pentose or hexuronic acid. Crystalline sialic acid from ovarian cyst fluid was obtained through the kindness of Dr. Odin, Uppsala.

Results. Ultracentrifugal analysis of the various fractions of serum separated by zone electrophoresis in a starch or polyvinyl supporting medium has demonstrated that the 19 S component observed in whole serum is distributed in two electrophoretic fractions with peak concentrations in the γ_1 and α_2 regions(13). A similar distribution is obtained when the serum is first subjected to repeated cycling in the preparative ultracentrifuge and then separated by electrophoresis. Fig. 1 shows the ultracentrifuge pattern obtained after concentration of the heavy components in the preparative ultracentrifuge. The sample contained less than 15% material with a sedimentation constant below 19. All the

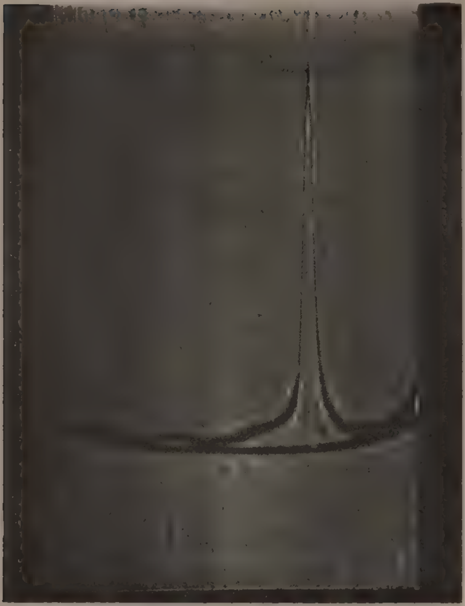

FIG. 1. Schlieren pattern obtained by ultracentrifuge analysis of a preparation of heavy component ($S_{20,w} = 19$ S).

albumin had been removed and only a small amount of 12 S and 7 S fractions remained. In this case no further albumin was added. The main peak after analysis at various concentrations was calculated to be $S_{20,w} = 19$ S. This sample was then subjected to zone electrophoresis in the starch medium and the protein pattern obtained is shown in Fig. 2. Two peaks are evident, one in the γ_1 and the other in the α_2 regions as indicated by comparison with the pattern of the original whole serum separated on the same starch block.

A number of different preparations of γ-globulin with varying amounts of heavy component were examined in the analytical ultracentrifuge and subjected to carbohydrate analysis. The amount of carbohydrate found was compared with the relative concentration of 19 S material. These results are plotted in Fig. 3. One of the major problems encountered in this work was progressive insolubility of the 19 S material after concentration and storage in the cold in the purified state. Associated with these alterations was the appearance of components with increased S rates in the ultracentrifuge pattern. Since these materials were formed at the expense of the 19 S peak, they were included in Fig. 3 as belonging in the 19 S class. The question whether all of these heavier components arose in this manner was not entirely answered. Trace amounts were also found in some freshly isolated γ-globulin preparations. No evidence was obtained that the major 7 S fraction of γ-globulin aggregated in this manner to material with an S rate higher than 12.

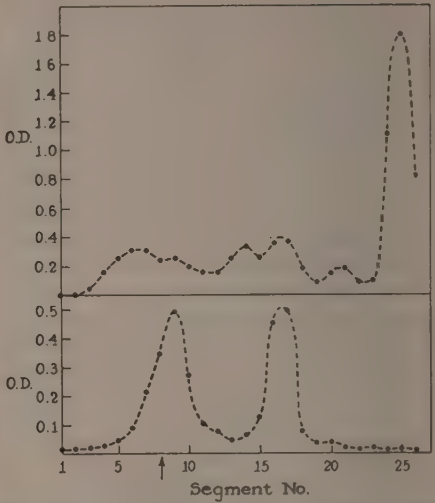

FIG. 2. Upper curve represents protein pattern obtained after electrophoretic separation of normal human serum. Lower curve represents a preparation of heavy component separated under identical conditions on the same starch block.

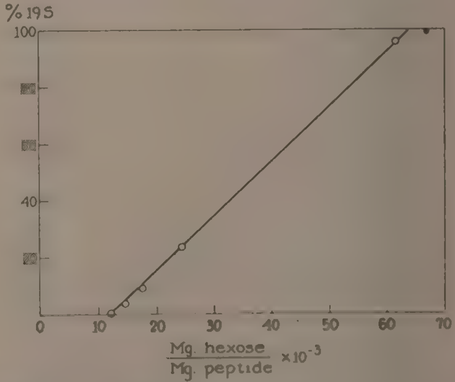

FIG. 3. Correlation between amount of 19 S material present in several γ-globulin preparations and the hexose-peptide ratio.

TABLE I. Carbohydrate Content of γ-Globulin and Various 19 S Proteins.

Protein	% N	% hexose	% fucose	% hexosamine	% sialic acid	% total carbohydrate	Hexosamine / Hexose
γ-glob. (7 S)	15.64	1.22	.29	1.14	.22	2.58	.94
γ_1 (19 S)	14.47	5.20	.62	2.90	1.70	9.80	.55
α_2 (19 S)	14.22	4.55		2.68	2.30	10.53	.59
Macroglob. I*	14.50	4.85	.62	2.66	1.71	9.22	.55

* Kindly furnished by Dr. H. F. Deutsch.

As shown in Fig. 3, there was a direct relationship between the percentage heavy components found in γ-globulin preparations and the carbohydrate-protein (peptide) ratio. The lowest point on the curve represents the ratio obtained for γ-globulin which is completely free of heavy components. The latter was obtained as previously described(7) from certain preparations of Fr. II-γ-globulin and from electrophoretic isolation of the slowly migrating material. The results obtained with 2 preparations which were close to 100% heavy component are indicated in the upper portion of the curve. The point, indicated as a solid dot, was not utilized in the plot because only a small amount of this preparation was available and the analyses were not considered to be as accurate as for the other points. Duplicate analyses were obtained for all the other preparations.

Table I shows the results of more detailed quantitation of the different carbohydrate components of one preparation which contained less than 5% of material with S rates lower than 19 S. For comparison the results obtained with 7 S γ-globulin and the α_2 heavy component are also indicated. It is apparent that the high molecular weight γ-globulin contains approximately 5 times as much hexose sugars as does the 7 S fraction. It has a low hexosamine-hexose ratio and is relatively rich in sialic acid. It is of interest that the α_2 fraction which is very similar to the γ_1 heavy component in size and shape(13) is also similar in carbohydrate content. Table I also gives the results obtained with a preparation of γ_1 material obtained from a case of Waldenström's macroglobulinemia. Immunological evidence obtained in this laboratory(14) and also by Korngold(15) has indicated a close relationship between the normal heavy component and the pathological macroglobulins. The carbohydrate content of this macroglobulin is similar to the normal preparation.

The sera of 4 patients (3 children and 1 adult) with agammaglobulinemia* were investigated to determine the concentration of the 19 S component migrating in the γ_1 region. When compared with normal sera a reduction to one-tenth or less of the normal level was found in 3 of the 4 cases. The methods used were not sensitive enough to indicate whether or not there was an even further reduction. Carbohydrate analyses on the fractions from one of these sera showed a complete absence of anthrone positive material throughout the γ_2 and γ_1 regions. The α_2 heavy component was not reduced in concentration in two of these sera in which it was examined.

Discussion. The high carbohydrate content of the 19 S group of proteins which migrate in the γ-globulin region demonstrates that these are not simple aggregates of the main 7 S type. They may represent polymerized molecules but if this is the case the basic units differ in hexose, hexosamine and sialic acid from other γ-globulins. Immunological studies which will be published separately (14) indicate that there are distinct antigenic differences between these 2 types of γ-globulin but that there is also considerable cross reaction. A similar immunological relationship was observed between 19 S and 7 S antibodies in horse sera by Treffers and associates(5). These observations suggest that the basic unit of the 19 S group of γ-globulins is similar to that of the 7 S class. The question of whether all the antigenic differences can be accounted for on the basis of the increased carbohydrate along with the large size of the heavy components is not as yet answered.

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* The authors are indebted to Dr. Robert Good for 3 of these sera.

It should be emphasized that the 19S γ globulin preparations analyzed in this study were never obtained 100% pure. Low molecular weight proteins could be almost completely removed and they presented a relatively minor problem. However the components with a sedimentation rate greater than 19S could never be eliminated entirely. The possibility remains that these materials may not be solely aggregates of the 19S fraction and cause slight alterations in the measured carbohydrate-protein ratio.

In the human, the blood group antibodies, certain abnormal red cell antibodies, and part of the Wasserman antibodies have been thought to be localized in the 19 S fraction of γ globulin. The possibility is raised that these and perhaps other antibodies are rich in carbohydrate. The increased carbohydrate, particularly sialic acid, would influence such properties as the isoelectric point and solubility and might confer unique characteristics to such antibodies.

The absence of detectable 19 S components of the γ_1-globulin type in the sera of 2 patients with agammaglobulinemia was reported previously(13). Further observations have confirmed these findings although small amounts were found in one case where some 7 S material was also present. These observations offer further evidence for a possible relationship between the heavy components under discussion and antibodies.

Summary. 1. The heavy components (19 S) have been highly concentrated from normal human serum by repeated cycling in the preparative ultracentrifuge. Electrophoresis of such preparations has permitted isolation of a high molecular weight γ-globulin fraction. 2. Analyses of this material indicate a high carbohydrate content with hexose sugars and sialic acid present at approximately 5 times the concentration found for the main 7 S γ-globulin. The hexosamine-hexose ratio is approximately 0.5. 3. Markedly reduced levels were found in the sera of 4 patients with agammaglobulinemia. The possibility that certain antibodies fall into the 19 S fraction and are similarly rich in carbohydrate is discussed.

1. Deutsch, H. F., Alberty, R. A., Gosting, L. J., and Williams, J. W., *J. Immunol.*, 1947, v56, 183.
2. Gordon, R. S., *ibid.*, 1953, v71, 220.
3. Davis, B. D., Moore, D. H., Kabat, E. A., and Harris, A., *ibid.*, 1945, v50, 1.
4. Kabat, E. A., *J. Exp. Med.*, 1939, v69, 103.
5. Treffers, H. P., Moore, D. H., and Heidelberger, M., *ibid.*, 1942, v75, 135.
6. Cann, J. R., *J. Am. Chem. Soc.*, 1953, v75, 4213.
7. Müller-Eberhard, H. J., and Kunkel, H. G., *J. Exp. Med.*, 1956, v104, 253.
8. Trautman, R., *J. Phys. Chem.*, 1956, v60, 1211.
9. Mokrasch, L. C., *J. Biol. Chem.*, 1954, v208, 55.
10. Dische, Z., and Shettles, L. B., *ibid.*, 1948, v175, 595.
11. Boas, N., *ibid.*, 1953, v204, 553.
12. Werner, I., and Odin, L., *Acta Soc. Med., Upsaliensis*, 1952, v57, 230.
13. Wallenius, G., Trautman, R., Franklin, E. C., and Kunkel, H. G., *Fed. Proc.*, 1956, v15, 378.
14. Franklin, E. C., and Kunkel, H. G., *J. Immunology*, in press.
15. Korngold, L., *Fed. Proc.*, 1956, v15, 597.

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Oxidation of Acetate and Malonate in Extrahepatic Tissues.* (22691)

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Previous studies from this laboratory have shown that a rapid "explosive-like" oxidation of acetate occurs in the extrahepatic tissues (1). To investigate the pathway of terminal oxidation of acetate, the ability of C^{14} acetate to be oxidized in the presence of malonate, a competitive inhibitor of succinic dehydrogenase, was tested. This procedure, which has been successfully applied to *in vitro* studies (2,3) and which has been reported effective in whole animal experiments(4,5), proved to be non-inhibitory to acetate oxidation at concentrations of malonate that were not lethal to the eviscerated animals. Radioactive mal-

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* This investigation was supported in part by research grant from the N.I.H., U.S.P.H.S.

TABLE I. Effect of Single Injection of Malonate on Oxidation of Radioactive Acetate. Results expressed as counts recovered in each period.

	Malonic acid administered	
	200 mg/kilo	250 mg/kilo
Period (30 min.)	Exp. 511*	Exp. 474*
1	4364	3350
2	5391	3350
3	3293	2925
4	5031	2925
5	4563	3020
6	4648	2640
Avg	4550	3035
7	3219	3120
8	4421	2860
9	4632	2500
10	5263	2600
11	4519	2800
12	5249	3140
Avg	4560	2837

* In Exp. 511 the acetate was inj. at rate of 100 mg/kilo/hr calculated as acetic acid. In Exp. 474 tracer doses of acetate were administered.

TABLE II. Oxidation of C^{14} Labeled Malonic Acid after Single Intrav. Inj. Results expressed as mg/kilo/hr of malonic acid except in Exp. 476 where oxidation is expressed as % of inj. counts. Carboxyl labeled malonate was used in Exp. 476, 481, 478 and 480; and malonate-2-C^{14} was used in Exp. 509.

	Exp. No.				
	476	481	478	480	509
		Amt of malonic acid inj., mg/kilo			
Hours	Tracer	50	300	685	200
1	5.0	3.0	7.9	18	2.7
2	8.5	3.4	13.4	17	3.3
3	6.5	2.8	13.5		2.9
4	5.9	1.9	8.1		3.5
5	3.7	1.5			3.7
Total % recovery	29.6	24	15		8
Total intracellular transfer of inj. dose (%)	58	55	60		

onate was therefore utilized to investigate this apparent non-inhibitory effect of malonate on acetate oxidation. The results obtained are reported here.

Methods. General treatment of animals. Non-fasted rabbits were eviscerated-nephrectomized(6) and maintained at normal blood sugar at all times. A tracheal cannula was inserted at the time of operation to collect the expired CO_2 for C^{14} determinations. *Procedure used in determining the effect of malonate on acetate oxidation (Table I).* Carboxyl-labeled C^{14}-acetate was administered as the sodium salt and free acid (1:1) by constant intravenous infusion for 3 hours. At the start of the fourth hour sodium malonate[†] was administered intravenously as a single dose. The infusion of radioactive acetate was continued as before for another 3-hour period without interruption. By this procedure each animal acts as its own control for acetate oxidation. In the absence of excess carrier acetate the rate of acetate oxidation is constant, provided that the rate of acetate injection is constant. *Oxidation of malonate (Table II).* C^{14}-labeled sodium malonate was injected at dosage levels (calculated as free acid) given in Table II. The expired CO_2 was collected each hour for C^{14} determinations. At the end of each experiment a plasma sample from each animal was also obtained for the C^{14} content. The total intracellular transfer of administered malonate was calculated from the counts administered and from the counts recovered in the terminal plasma samples. The extracellular space was assumed to be 22% of body weight. *Volume of distribution of injected malonate (Table III).* Plasma samples were taken at regular intervals after intravenous administration of radioactive malonate. These plasma samples were oxidized and the carbon collected and counted as $BaCO_3$.

Results. Effect of malonate administration on oxidation of acetate. It is apparent from the data in Table I that malonate ad-

TABLE III. Volume of Distribution of Injected Carboxyl Malonate. Results expressed as % of body wt (corrected for oxidation).

		Amt of malonic acid inj., mg/kilo	
	Tracer	50	300
Time	Exp. 476	Exp. 481	Exp. 478
20 min.	40	20	18
1 hr 20 min.	48	29	21
2 20		28	28
3 20	54	43	
4 20		33	37
5 20	65	34	

[†] Carboxyl labeled C^{14} disodium malonate was prepared from diethylmalonate purchased from Tracerlab, Inc. Methylene labelled C^{14} disodium malonate was prepared from dicalcium malonate purchased from Volk Radiochemical Co., Chicago, Ill.

ministration in doses up to 250 mg/kilo does not inhibit oxidation of either tracer or carrier amounts of acetate.

Oxidation of malonate. The data in Table II show that both carboxyl and methylene C^{14}-labeled malonate are oxidized to CO_2 by the eviscerated animal. Rate of oxidation appears to be dependent on plasma concentration of malonate since the highest oxidation rate is found in those animals administered malonate at the higher dosage level. It should be pointed out that even in the animal with the highest malonate oxidation rate, the oxidation of malonate represents less than 2% of total metabolism of the animal. Intracellular transfer rate, which ranges from 55 to 60% over the 5-hour period of observation, suggests that cell entry is an important limiting factor in the rate of malonate oxidation. Dosages of malonate at 685 mg/kilo and higher proved to be quite toxic to the animal. This toxicity did not appear to be due to an inhibition of the citric acid cycle, but rather to the possible binding of metal ions by malonate. One indication of this was the lack of blood coagulation in the eviscerated animals treated with malonate.

Volume of distribution of injected malonate. The data in Table III show that injected malonate circulates initially only in a volume equivalent to the extracellular compartment. The intracellular transfer rate of malonate increases slowly with time. We consider the extracellular compartment to be about 20-25% of body weight.

Discussion. It has been shown (Table I) that under our conditions malonate does not have any apparent effect on oxidation of acetate to CO_2 by the eviscerated-nephrectomized rabbit. The lack of effect of even higher doses of malonate on acetate oxidation had been noted previously in the intact rat(7). The inability of malonate to inhibit acetate oxidation can be explained by 2 findings presented in this paper. First, volume of distribution studies show that cell permeability barriers exist for malonate, for approximately 40-50% of the injected dose is still circulating in the extracellular compartment 5 hours after malonate administration. Next, oxidation studies demonstrate that both carboxyl and methylene carbons of malonate can be oxidized. Thus, it can be seen that a slow rate of malonate entry into the cells coupled with its oxidation by the tissues, prevents intracellular concentration of malonate from attaining a level high enough to inhibit a succinic dehydrogenase.

The ability of the eviscerated animal to oxidize malonate is not surprising. Lifson and coworkers(8,9) studying C^{13} carboxyl labeled malonate conversion in the intact mouse and rat demonstrated the degradation of malonate to carbon dioxide. More recently Hayaishi(10) has reported that rat kidney homogenates were capable of producing isotopic CO_2 from radioactive malonate. Homogenates from rat liver and muscle, however, were found to be inactive. From our results it is clear that tissues other than kidney are also able to oxidize malonate.

A finding of particular interest to us is that a concentration of malonate much less than that normally used to demonstrate malonate inhibition in intact animals, was found to be lethal to the eviscerated animals. If one assumes that administration of this compound results in a chelation of metal ions, then it would appear that the *in vivo* administration of malonate could result in non-specific metabolic alterations which would allow very little experimental value for elucidating metabolic pathways. The finding of succinate(5) and glucose accumulation(4) by others after malonate injection could very well be a demonstration of malonate inhibition in the classical sense; however, this accumulation may also be due to causes other than a strict inhibition of succinic dehydrogenase.

Summary. 1. Oxidation of acetate to CO_2 by the eviscerated-nephrectomized rabbit is unaffected by malonate. 2. Studies on the fate of carbon-14 labeled malonate indicate that the intracellular transfer rate of malonate is low. 3. Conversion of radioactive malonate to CO_2 has been demonstrated in the eviscerated-nephrectomized rabbit. 4. The slow rate of cell entry and subsequent oxidation of malonate is offered as a possible explanation for the non-inhibitory effect of malonate on acetate oxidation.

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1. Wick, A. N., and Drury, D. R., *J. Biol. Chem.*,

1952, v199, 127.

2. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, 1931, v25, 117.
3. Krebs, H. A., and Eggleston, L. V., *ibid.*, 1940, v34, 442.
4. Handler, P., *J. Biol. Chem.*, 1945, v161, 53.
5. Busch, H., and Potter, V. R., *ibid.*, 1952, v198, 71.
6. Drury, D. R., *Am. J. Physiol.*, 1935, v111, 289.
7. Busch, H., and Potter, J. R., *Cancer Research*, 1953, v13, 168.
8. Lifson, N., and Stolen, J. A., PROC. SOC. EXP. BIOL. AND MED., 1951, v74, 451.
9. Lee, J. S., and Lifson, N., *J. Biol. Chem.*, 1951, v193, 253.
10. Hayaishi, O., *ibid.*, 1955, v215, 125.

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Cultivation of Type 2 Dengue Virus in Rhesus Kidney Tissue Culture.* (22692)

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It was reported(1) that mouse-adapted type 1 dengue virus of the Mochizuki and Hawaiian strains multiplied in trypsinized rhesus kidney tissue cultures, and produced a cytopathogenic effect. It was also demonstrated that degeneration of infected cells was inhibited by type specific anti-dengue immune rabbit serum, thus making possible virus neutralization tests in tissue culture. In the present paper, experiments are reported in which type 2 dengue virus was cultivated in tissue culture.

Materials and methods. Tissue cultures employed consisted of trypsinized rhesus kidney cells(2). Technical details including preparation and incubation of cultures, constituents of medium, etc., were described previously(1). Type 2 dengue virus of the New Guinea C strain was received on Jan. 25, 1955, from Dr. Edwin H. Lennette in the form of 20% mouse brain homogenate in 30% normal rabbit serum frozen with dry ice. It was stated that this material represented the 19th mouse brain passage, and that its LD_{50} titer was $10^{-6.2}$ at time of harvest, Nov. 11, 1953. The virus was kept in dry ice chest until used. On Feb. 1, 1955, the frozen material was thawed at room temperature, and diluted with Hanks' balanced salt solution. Its infectivity was measured by injecting 2 to 3-week-old white mice intracerebrally with 0.02 ml of 10-fold dilutions. The mortality ratios of injected mice were 4/4 for the 10^{-3} dilution and 0/4 each for dilutions of 10^{-4} through 10^{-8}.

Results. Serial passage through tissue culture: Seven cultures were inoculated with 0.2 ml of above-mentioned virus diluted 10^{-3}. After 10 minutes 1.8 ml of culture medium was added to each tube. The cultures were incubated in the stationary state at 35°C. Half or whole volume changes of medium were made usually every 2 to 3 days, to keep the pH of the fluid phase in the range of 7.2 to 7.6. Eleven days after beginning of incubation a portion of culture fluid was taken from each tube and pooled with fluids from other similar tubes. 0.2 ml of the mixture was then inoculated into new cultures with subsequent addition of 1.8 ml of medium to each tube. In this manner serial passages through tissue cultures were performed at intervals of 7 to 15 days. The virus content of the inoculum for each subculture was assayed by injecting 0.02 ml of 10-fold dilutions into mice. The results obtained are summarized in Table I. The specimens used for tissue culture passage and virus titration contained no agent cultivable on ordinary bacteriological culture media.

The virus was cultivated in this tissue culture system for 18 passages during 176 days.

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TABLE I. Serial Transmission of Type 2 Dengue Virus of New Guinea C Strain through Rhesus Kidney Tissue Culture.

Passage in tissue culture	Mortality ratios of mice inoculated with tissue culture fluid diluted 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Cumulative period of cultivation, days	Calculated dilution of original infected mouse brain based on passage and medium-change
1	2/3	1/3*	2/3	1/3	0/3	0/3	11	$10^{-4.0}$
2	3/3	2/3	0/3*	1/3	0/3	0/3	25	$10^{-6.8}$
3	0/3	0/3	0/3	0/3	0/3	0/3	38	$10^{-8.7}$
4	2/3	2/3	1/3	1/3	0/3	0/3	50	$10^{-12.0}$
5	1/3	0/3	2/3	1/3	0/3	0/3	65	$10^{-13.8}$
6	2/3	1/3	1/3	0/3	0/3	0/3	74	$10^{-15.2}$
7	0/3	1/3	1/3	0/3	0/3	0/3	81	$10^{-16.5}$
8	2/3	2/3	1/3	0/3	0/3	0/3	92	$10^{-18.1}$
9	1/3	0/3	2/3	0/3	0/3	0/3	100	$10^{-19.4}$
10	1/3	2/3*	0/3*	1/3	0/3	0/3	110	$10^{-21.8}$
18	3/3	2/3	0/3	2/3	1/3	0/3	176	$10^{-41.5}$†

* One of the surviving mice showed paralytic signs and recovered.

† For 1 ID_{50} in 18th passage.

One ID_{50} in the 18th passage represented a dilution of $10^{-41.5}$ of the original infected mouse brain homogenate. As reported by other investigators(3), mortality ratios of mice inoculated with the New Guinea C strain virus were erratic.

Morphological changes: Inoculated cultures were examined with ordinary light microscope. They showed cellular degeneration which was indistinguishable from that produced by type 1 dengue virus(1). Five days to one week after inoculation of virus, the cells were dark and spherical. This was followed by their detachment from the glass surface. After about 2 weeks only a relatively small part of the cell population remained. After 3 to 4 weeks practically all cells had disappeared. It was confirmed again in our experiments that uninoculated control cultures never showed widespread destruction of cells; changes resulting from prolonged incubation were limited. A large population of cells of normal appearance remained in uninfected cultures incubated 4 weeks or more.

Parallel titrations of virus in mice and in tissue cultures: The titers of cultivated virus were compared in mice and in tissue cultures. The inoculum was 0.02 ml for mice by the intracerebral route, and 0.2 ml for tissue cultures containing 1.8 ml of medium. The results obtained are given in Table II. In these limited tests the highest dilution which caused death of mice was greater than that producing definite degeneration of tissue culture cells.

Neutralization test in tissue culture: Immune serum was obtained by injecting a rabbit repeatedly with the New Guinea C strain of virus that had been propagated in mouse brain and had never been passed in tissue culture. The procedure for immunization was similar to that used in obtaining anti-type 1 immune serum(1). Serum collected from the rabbit prior to immunization served as a control non-immune serum. The serum was inactivated by heating at 56°C for 30 minutes, and stored at —10°C until used. Type 1 Mochizuki and Hawaiian strains of virus and

TABLE II. Parallel Titrations of Type 2 Dengue Virus of the New Guinea C Strain in Mice and in Tissue Cultures.

Passage in tissue culture	Inoc. into*	Dilution of infected tissue culture fluid 10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
10	Mice		1/3†	2/3	0/3	1/3	0/3	0/3
	Tissue cultures		3/3†	3/3	0/3	0/3	0/3	0/3
18	Mice	1/3	3/3	2/3	0/3	2/3	1/3	0/3
	Tissue cultures	3/3	3/3	3/3	2/3	0/3	0/3	0/3

* Inoculum: 0.02 ml in mice intracer., and 0.2 ml in tissue cultures with 1.8 ml of medium.

† Numerators indicate No. of mice showing characteristic signs of infection or tissue culture tubes showing definite degeneration. Denominators indicate No. of mice or tissue culture tubes inoculated.

TABLE III. Neutralization Tests in Tissue Cultures of Type 1 and Type 2 Dengue Viruses.

Virus					Serum*		
Type	Strain	Passage in tissue culture	ID_{50} for tissue culture/0.2 ml	Dilution of infected tissue culture fluid	Anti-Mochizuki immune	Anti-New Guinea C immune	Control†
1	Mochizuki	43	$10^{-5.0}$	100-fold	0/5‡	5/5	5/5
	Hawaiian	25	$10^{-5.25}$	”	0/5	5/5	5/5
2	New Guinea C	18	$10^{-8.25}$		5/5§	0/5	5/5

* Diluted 1:5 with 0.1% lactalbumin hydrolysate medium. Aliquots of this mixed with equal volumes of virus suspension.

† Taken before immunization from a rabbit used for immunization against New Guinea C strain virus.

‡ Numerators indicate No. of tissue culture tubes showing definite degeneration. Denominators indicate No. of tissue culture tubes inoculated. The culture fluid used for changing medium included 1% of immune or control serum.

§ Degeneration of tissue cultures inoculated with mixture of type 2 virus and anti-type 1 serum was milder than that in control cultures exposed to type 2 virus and non-immune serum.

anti-Mochizuki immune rabbit serum were used to determine heterologous reactions. In performing the neutralization tests a given concentration of cultivated virus was mixed in equal volume with serum diluted 1:5 in 0.1% lactalbumin hydrolysate medium. After incubation at 37°C for 1 hr and at 4°C for 1 more hr, 0.2 ml of the mixture was inoculated into tissue culture tubes with subsequent addition of 1.8 ml of medium. Examples of results obtained are shown in Table III.

It was demonstrated that the viruses were neutralized by the homologous serum, but not by the heterologous serum. It appeared, however, that the degeneration shown in tissue cultures inoculated with the mixture of type 2 virus and anti-type 1 serum was a little milder than that shown in control cultures exposed to virus and non-immune serum.

Summary and conclusions. 1) Mouse-passaged type 2 dengue virus of the New Guinea C strain was cultivated in trypsinized rhesus kidney tissue cultures grown directly on glass surfaces and incubated in the stationary state at 35°C. The virus was maintained in this system of tissue culture through 18 passages during 176 days. Culture fluid from the 18th passage representing a $10^{-41.5}$ dilution of the original infected mouse brain was infective for mice. 2) The infected culture cells exhibited characteristic degeneration indistinguishable from that associated with infection of tissue cultures with type 1 dengue virus. Infectivity of the virus was neutralized by specific antiserum as indicated by the fact that the degeneration was suppressed completely by homologous immune rabbit serum, but not by heterologous serum. It appeared that the cellular degeneration produced by type 2 virus mixed with anti-type 1 serum was a little milder than that produced by the same virus mixed with the control non-immune serum. Although further studies are necessary to clarify the antigenic relationships of type 1 and type 2 dengue viruses, it is possible that the reduced degeneration was due to a partial neutralization by heterologous antibody.

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1. Hotta, S., and Evans, C. A., *J. Infect. Dis.*, 1956, v98, 88.

2. Youngner, J. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 202.

3. Meiklejohn, G., England, B., and Lennette, E. H., *Am. J. Trop. Med. and Hyg.*, 1952, v1, 51.

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Anaerobic Loss of Endogenous Glycogen in Rat Heart Slices. (22693)

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In the course of a series of comparative studies it was found that rapid depletion of the endogenous glycogen of rat heart occurred *in vitro* under anaerobic conditions. Attempts to demonstrate an accumulation of lactic acid under these conditions were unsuccessful and lactic acid was actually found to disappear slowly during anaerobic incubations. A summary of typical data appear in Table I. No significant concentration of preformed or 7 minute hydrolyzable reducing substances, pyruvate, acetoin, citrate, volatile acids or triose phosphates was found in any time period. Small concentrations of fructose and high concentrations of hexosemonophosphate were demonstrated in all time periods. Very high concentrations of 5 hour hydrolyzable reducing substances of undetermined origin were found in all time periods but wide variations between individual experiments were also found: 898 μg/100 mg (542-1131) at zero time, 288 (107-392) at 10 minutes, and 760 (733-851) at 30 minutes. On the basis of these results the following compounds are thought to be excluded as end products of the observed glycogen breakdown in heart slices: glucose, hexose phosphates and diphosphate, fructose, glyceraldehyde phosphate and dihydroxyacetone phosphate, pyruvate and lactate, acetoin, acetate and citrate.

An attempt was made to evaluate this unusual finding in the whole animal. To simulate the anaerobic *in vitro* incubations the heart was allowed to remain within the chest for varying times after the rat was beheaded. Under these conditions the endogenous glycogen of the heart fell rapidly but there was a concurrent accumulation of lactic acid, in agreement with the usual glycolytic theory (Table II).

The "extra" lactate formed in this system probably represents that formed in the terminal metabolism from the various glycolytic intermediates.

Discussion. A survey of the literature failed to reveal a comparable study. In a series of reports in 1932-35, Haarman indicated that glycogen added to heart *brei* was destroyed but that lactic acid was not formed (15,16). From our experiences with attempting to prepare a heart brei it seems that Haarman's work should be considered a study of a variety of isolated soluble enzyme systems from heart. Barron, *et al.*(17) have shown that anaerobic glycolysis in rat heart slices which have been thoroughly oxygenated before incubation leads to an accumulation of lactic acid as indicated by chemical analysis and CO_2 production from the buffer. This apparent contradiction of our results is probably the result of different pre-treatments of the slices. In the present study the slices were collected in nitrogen-CO_2-gassed buffer rather than in oxygen-gassed buffer. The demonstration that hexosemonophosphate accumulates in the skeletal muscle of rats killed by decapitation or similar means(18), led us to believe that hexosemonophosphate was the accumulated end product of the observed glycogen breakdown. Determination of the hexosemonophosphate content of the heart slices at the various time periods showed a high concentration but no accumulation of this intermediate. The possibility that the failure of lactic acid to accumulate may reflect the operation of an alternate pathway of carbohydrate metabolism, such as those known for hexose(19) and pyruvate(20), has not been investigated. The report(17) that lactic acid accumulates as expected when the tissue has been thoroughly oxygenated or, as here, when incubation of the tissue takes place *in situ*, leaves some question as to the physiological significance of such a pathway.

Experimental. Five- to six-month-old Supplee white rats of both sexes were used. All preparations were carried out at 0-3° unless otherwise indicated.

In vitro incubations: The rats were beheaded and the heart removed immediately

TABLE I. Disappearance of Glycogen and Lactic Acid *In Vitro*.

Sample	Elapsed time, min.	Glycogen, μg/100 mg*	Lactic acid, μg/100 mg	CO_2, μl/flask
Pre-equilibration	0	273 (8)	75 (8)	
Post-equilibration	10	138 (4)	57 (4)	
Post-incubation†	40	20 (4)	46 (4)	0 (24)

* Wet wt.

† Incubations at 25° or 38°. Data typical of either temperature. Numbers in parentheses are No. of separate analyses or observations.

to an appropriately gassed buffer solution (pH 7.4 Krebs Ringer bicarbonate or phosphate (for acetate study) buffers(9)). After about 10 seconds the heart was removed, the auricles and major vessels trimmed off, and 0.5 mm slices prepared with a Stadie slicer (10). The first slice was discarded and the others placed in a fresh gassed buffer solution. The slices were blotted dry on filter paper and 100-300 mg (depending on the experiment) transferred to 3 ml of gassed buffer in standard Warburg vessels. The vessels were equilibrated at 25°C or 38°C under flowing nitrogen-CO_2 (95%-5%) or nitrogen (acetate study) for 10 minutes and then incubated 30 minutes by the standard Warburg technic. The reaction was stopped by the addition of 0.3 ml 35% trichloroacetic acid (TCA) or 0.3 ml 24 N H_2SO_4 (acetate study). The flask contents were then homogenized, centrifuged (except acetate samples) and the supernatant analyzed.

In situ incubations: Rats were beheaded and the heart removed from the chest at the indicated time to gassed buffer solution. The washed heart was cut in half and one-half homogenized in a fresh buffer solution containing TCA. Lactate analyses were performed on the supernatant while glycogen was determined on the remaining half heart.

The following analytical methods were employed: glycogen(11), lactate(12), glucose and reducing substances(13,14,15), fructose (16), hexosemonophosphate(17), pyruvate (18), acetate(19), acetoin(20), triosephosphates(21) and citrate(22).

TABLE II. Changes in Glycogen and Lactic Acid *In Situ*.

Time after beheading, min.	Glycogen, μg/100 mg	Lactic acid, μg/100 mg	% recovery (glycogen as lactate)
0	249	84	
2	196	175	
5	196	184	
10	46	306	
30	39	335	119.8%

Summary. An anomalous situation has been encountered in a study of the endogenous carbohydrate metabolism of heart. It is believed that this anomaly, the failure of lactic acid to accumulate during anaerobic glycogen breakdown, may reflect the operation of an alternate pathway.

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1. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, 2nd Edit. Burgess Publishing Co., Minneapolis, 1949, p119.

2. Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, 1944, v154, 687.

3. Good, C. A., Kramer, H., and Somogyi, M., *ibid.*, 1933, v100, 485.

4. Potter, V. R., editor, *Methods in Medical Research*, v1, Yearbook Publishers, Inc., Chicago, 1948, p345.

5. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

6. Somogyi, M., *ibid.*, 1952, v195, 19.

7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *op. cit.*, p188.

8. Roe, J. H., Epstein, J. H., and Goldstein, N. P., *J. Biol. Chem.*, 1949, v178, 839.

9. Cori, G. T., and Cori, C. F., *ibid.*, 1932, v94, 561.

10. Koepsell, H. J., and Sharpe, E. S., *Arch. Biochem. Biophys.*, 1952, v38, 443.

11. Serlin, I., and Cotzeas, G. C., *J. Biol. Chem.*, 1955, v215, 263.

12. Westerfeld, W. W., *ibid.*, 1945, v161, 495.

13. Sibley, J. A., and Lehninger, A. L., *ibid.*, 1949, v177, 859.

14. Natelson, S., Pincus, J. B., and Lugovoy, J. K., *ibid.*, 1948, v175, 745.

15. Haarman, W., *Biochem. Z.*, 1932, v225, 103.

16. Haarman, W., and Brink, H., *ibid.*, 1935, v282, 419.

17. Barron, E. S. G.; Sights, W. P., and Wilder, V., *Arch. exp. Path. u. Pharmakol.*, 1953, v219, 338.

18. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1931, v94, 581.

19. Cohen, S. S., in *Chemical Pathways of Metabolism*, (D. M. Greenberg, Editor), Academic Press Inc., New York, 1954, vI, pp173-234.

20. Groth, D. P., LePage, G. A., Heidelberger, C., and Stoesz, P. A., *Cancer Research*, 1952, v12, 529.

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Effect of N-Allylnormorphine Upon Massive Doses of Narcotic Drugs. (22694)

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Although N-allylnormorphine (nalorphine) is an effective antagonist against a wide spectrum of pharmacological actions of opiates (1,2), and has proven to be clinically useful in cases of narcotic overdosage(3-6), Koppanyi and Karczmar(7) did not succeed in saving mice from lethal doses of morphine by single injections of nalorphine. On the other hand, Unna(8) reported that nalorphine in divided doses reduced mortality in mice receiving 800 mg/kg of morphine, and Gruber (9) showed that even a single dose of the antagonist was protective against morphine sulfate up to 800 mg/kg if the ratio of the two drugs was properly chosen.

The present experiments demonstrate a difference in the protective effect of nalorphine against lethal doses of various opiates and a species difference in the responses.

Experimental. Preliminary experiments were performed in rabbits receiving meperidine hydrochloride by slow intravenous injection. The dose of meperidine used was 25 mg/kg, except in two rabbits in which severe convulsions forced discontinuance of the injection at 15 and 20 mg/kg, respectively. The results (Groups I-III, Table I) show that nalorphine, administered subcutaneously 30 minutes before the opiate, was in no case successful in preventing convulsions. In each case where death occurred, respirations ceased at about the same time convulsions set in. Respirations were maintained, however, in the meperidine-injected rabbits pre-treated with 10 mg/kg of nalorphine (Group III). In these, the convulsant action of the opiate persisted for 4 to 10 minutes, and the animals then recovered. Two animals (Group IV) receiving the anticonvulsant agent, phenobarbital, in addition to nalorphine exhibited neither convulsions nor grossly visible respiratory depression when 25 mg/kg of meperidine was injected.

These preliminary observations in rabbits suggested that nalorphine antagonized the respiratory depressant effect of the opiate without markedly influencing the convulsant activity. In Group III, sufficient nalorphine was administered so that respiration did not stop and the animals survived, but the convulsions were as severe as in the previous groups. It appeared, then, that convulsant activity might be one effect of opiates that nalorphine would not antagonize. In order to test this further, experiments were conducted in mice and rats.

For these experiments, male IS32 mice of about 20 g body weight and male Holtzman rats of about 130 g were employed. Table II

TABLE I. Effect of N-Allylnormorphine upon Convulsant and Lethal Effects of Intravenous Meperidine in Rabbits.

Group	Antidote	No. of animals	No. convulsing	No. surviving
I	None	2	2	0
II	Nalorphine, 1 mg/kg	2	2	0
III	Nalorphine, 10 mg/kg	4	4	4
IV	Nalorphine, 10 mg/kg + phenobarbital, 100 mg/kg	2	0	2

TABLE II. Protection of Mice by Nalorphine Hydrochloride against Lethal Doses of Morphine or Methadone.

Morphine sulfate			Methadone hydrochloride		
	No. dead/total			No. dead/total	
Dose, mg/kg	Without nalorphine	With nalorphine*	Dose, mg/kg	Without nalorphine	With nalorphine†
350	6/10	0/10	25	3/10	0/10
440	7/10	2/10	29.5	7/10	0/10
555	7/10	1/10	35	8/10	0/10
700	10/10	5/10	41	8/10	0/10
			50	9/10	0/10
			70	10/10	2/10

* Nalorphine HCl dose: 25 mg/kg, repeated at ½- to 1-hr intervals until total of 225 mg/kg had been given.

† Nalorphine HCl dose: 10 mg/kg 15 min. before methadone, repeated 1 hr and 6 hr after methadone.

demonstrates that, contrary to the findings of Koppanyi and Karczmar (*loc. cit.*), adequate doses of nalorphine offer considerable protection to mice receiving lethal doses of morphine. It is evident, however, that nalorphine is more effective against methadone than against morphine in mice. One difference was observed between the mice receiving morphine and those receiving methadone: These high doses of morphine produced convulsions in the animals, while methadone did not. The results are therefore consistent with the finding in rabbits that nalorphine is less effective against convulsions than against respiratory depression.

Codeine phosphate and prisilidene hydrochloride are convulsant drugs at toxic dose levels in mice, but not in rats. In the latter species, hypnosis, catalepsy and respiratory depression are the prominent effects of toxic doses of these drugs, and convulsions do not develop except terminally (probably asphyxial in origin). In Table III, it is shown that rats, but not mice, are easily protected by nalorphine against lethal doses of these drugs.

Another experiment therefore was performed in which an anticonvulsant drug was included. Forty mice were divided into 4

TABLE III. Species Difference in Response of Rats and Mice to Nalorphine Hydrochloride when Given Lethal Doses of Codeine or Prisilidene.

Mice			Rats		
	No. dead/total			No. dead/total	
Dose, mg/kg	Without nalorphine	With nalorphine*	Dose, mg/kg	Without nalorphine	With nalorphine†
		A. Codeine phosphate			
300	3/10	3/10	500	7/10	0/10
330	3/10	4/10	700	5/ 5	0/ 5
363	2/10	9/10	1000	5/ 5	0/ 5
400	9/10	10/10			

* Nalorphine HCl dose: 25 mg/kg immediately before codeine, repeated ½ hr later. Death occurred before a third dose could be given.

† Nalorphine HCl dose: 25 mg/kg ½ hr before codeine, repeated 1 hr and 4 hr after codeine.

B. Prisilidene hydrochloride

Mice			Rats		
50	0/10	0/10*	40	5/5	0/5†
72	1/10	2/10	60	4/5	0/5
104	7/10	5/10	80	5/5	0/5
150	9/10	10/10			

* Nalorphine HCl dose: 25 mg/kg repeated at ½-hr intervals to a total of 200 mg/kg.

† Nalorphine HCl dose: 25 mg/kg—no repetition.

TABLE IV. Effect of Combination of Anticonvulsant Drug and Nalorphine in Mice Receiving Lethal Dose of Codeine.

			Effect of codeine PO_4 (450 mg/kg)	
			No. animals	
Group	No. of animals	Pretreatment	Convulsing	Dead
I	10	Phenobarbital Na, 50 mg/kg	2	9
II	10	Nalorphine HCl, 50 mg/kg	10	8
III	10	Combination	1	1
IV	10	None	10	10

groups, with drugs injected subcutaneously as follows: *I*. Phenobarbital sodium 50 mg/kg. *II*. Nalorphine hydrochloride 50 mg/kg. *III*. Both phenobarbital and nalorphine, each 50 mg/kg. *IV*. Uninjected controls. One-half hour later, the animals received codeine phosphate 450 mg/kg, a dose slightly above the LD_{100} according to the data in Table III. The results of this experiment are shown in Table IV. It is apparent that phenobarbital alone controlled the convulsions in most of the mice, but did not prevent death. Nalorphine alone failed to control the convulsions, and saved the life of only 2 of 10 mice. When both nalorphine and phenobarbital were given, convulsions and death were prevented in 9 of 10 animals.

Discussion. It appears from these experiments that not all of the effects of opiates are equally well inhibited by nalorphine. One effect in particular seems to be poorly antagonized, and that is the convulsant action. The tendency of narcotic analgesic drugs to produce convulsions varies among drugs and among species. For example, large doses of morphine are convulsant in some species, but convulsions are rare in man(10). Our results indicate that codeine and prisilidene are highly convulsant for mice, but not for rats. If one assumes that nalorphine competes with morphine-like drugs at their sites of action, it is possible that it has less affinity for sites responsible for convulsions than do the convulsant opiates.

Summary. Nalorphine is an effective antidote for doses of opiates which would otherwise be lethal, in those instances where signs of depression are the dominant toxic manifestations. It is relatively ineffective, however, in those instances where convulsions are a prominent feature. In rabbits and mice receiving convulsant doses of meperidine, codeine or prisilidene, nalorphine alone was seldom life-saving, but administration of both phenobarbital and nalorphine adequately protected the animals. Codeine and prisilidene did not produce convulsions in rats; nalorphine alone was an effective antidote in these instances.

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1. Winter, C. A., Orahovats, P. D., Flataker, L., Lehman, E. G., and Lehman, J. T., *J. Pharmacol. and Exp. Therap.*, 1954, v111, 152.

2. Winter, C. A., Gaffney, C. E., and Flataker, L., *ibid.*, 1954, v111, 360.

3. Eckenhoff, J. E., Hoffman, G. L., and Dripps, R. D., *Anesthesiology*, 1952, v13, 242.

4. Fraser, H. F., Wikler, A., Eisenman, A. J., and Isbell, H., *J. Am. Med. Assn.*, 1952, v148, 1205.

5. Finestone, A. J., and Ekterowicz, F. C., *Am. Pract.*, 1953, v4, 640.

6. Strober, M., *J. Am. Med. Assn.*, 1954, v154, 327.

7. Koppanyi, T., and Karczmar, A. G., *Fed. Proc.*, 1953, v12, 337.

8. Unna, K., *J. Pharmacol. and Exp. Therap.*, 1943, v79, 27.

9. Gruber, C. M., Jr., *ibid.*, 1954, v111, 404.

10. Krueger, H., Eddy, N. B., and Sumwalt, M., *Supp. No.* 165, *U. S. Public Health Reports*, 1946.

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Cyclic Variation in the Mule Deer Thymus.* (22695)

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A series of fawn, yearling, and adult female mule deer (*Odocoileus hemionus hemionus*) was collected on the National Bison Range, Moiese, Mont. for purposes of studying the reproductive cycle of the female mule deer. Specimens of each of the 3 age groups were killed each month between Aug., 1953 and Sept., 1954. Measurements, tissue weights, and tissue preservation were made in the field. One of the tissues routinely measured, and portions preserved, was the bilobed thymus gland.

Method. The female deer were hunted, shot, and autopsied on the open range. All measurements were made on the fresh thymus *in situ*, each lobe being measured separately. The length, width at the middle, and the thickness in the center transections were taken with a celluloid rule. A portion of each gland was removed and preserved in Bouin's fixative. No histological study of this material has been made to date. The volume of each gland was calculated by assuming the gland to be an elliptical spheroid. The calculated volumes of the 2 lobes were averaged and divided by the total body weight of the animal to obtain the thymus volume/body weight ratios used in Fig. 1. The curve of the thymus volume/body weight represents the combined average values of the three age classes for each month. The total number of animals used was: 22 fawns, 22 yearlings, and 26 adult female mule deer.

Results. All 3 classes exhibited the same type of annual thymus volume cycle, although the fawns had the highest value of the 3 age classes for the late spring-early summer months. The pregnant adult does had the least high value of the 3 age classes for that time of year, and the yearlings had intermediate values. All 3 age classes were equally low during the winter months whether they were pregnant or non-pregnant.

The thymus gland is commonly used as an indicator of systemic stress(1), but it seemed conjectural to us whether or not in this case the thymus gland could be used as an indicator of systemic stress. The annual variation in thymus gland volume might well represent stress factors of high temperatures and "burnt up" vegetation in the summer months, and cold temperatures and difficulty of obtaining feed in the snowy winter months. The slower recovery of thymus volumes in the yearlings and adults in the early spring can be attributed to placental hormones from December to June. Non-pregnant fawns had thymi with values as low as the pregnant yearlings and adults during December, January, and February.

The data also permit the hypothesis that light stimulates the production of a thymotropic hormone via the pituitary gland. This hypothesis seems as reasonable as that of stress when the close correlation is noted between the average monthly hours of daylight and the thymus volume/body weight curves, (Fig. 1). Also there was no indication of annual adrenal cycle in weight, volume, or cortex-medulla ratio which is believed to be intimately involved in any symptoms of systemic stress.

The apparent relationship of the thymus volume in the mule deer to climatic conditions revealed in this study warrant the attention of other investigators. This is particularly appropriate since circumstances prevent the present authors from immediately following up the ramifications of the problem on either domestic, laboratory, or game animals.

Summary. Thymus glands from a series of fawn, yearling, and adult female mule deer, collected on the National Bison Range, Moiese, Mont., varied in volume with the season. The thymus reached its lowest volume during the winter months (Dec.-Feb.)

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* This investigation was made possible through the cooperation of the U. S. Fish and Wildlife Service, the Montana Fish and Game Department, and the Montana Cooperative Wildlife Research Unit.

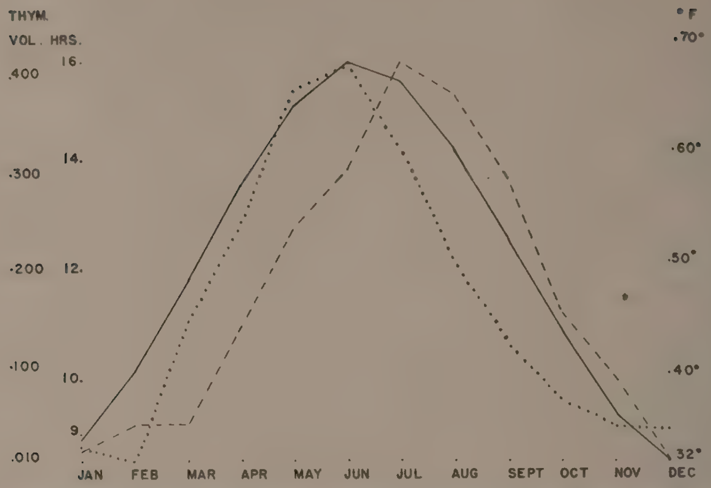

FIG. 1. Annual weather cycle, and thymus volume of female mule deer, National Bison Range, 1953, 1954. Avg monthly day length, ———; avg monthly temperature, 1953-1954, – – – – –; avg monthly thymus vol/body wt ratios of female mule deer of all age groups, • • • • •

and reached its largest size in all 3 age classes during late spring and early summer (May-July). All 3 groups exhibited the same type of annual thymus volume cycle, although the fawns had the highest value of the 3 age classes for the late spring-early summer months. Pregnant or lactating adult does had the lowest value of the 3 age classes for that time of year, although these values were considerably higher than those of the winter months. The data permit the hypothesis that light stimulates production of a thymotropic hormone probably via the pituitary gland. There is very good correlation between average monthly hours of daylight and thymus volume/body weight curves. Stress factors such as high temperatures and "burnt up" vegetation in late summer, and cold temperatures and difficultly available food in snowy winter months may also affect the size of the thymus.

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1. Selye, Hans, *The physiology and pathology of exposure to stress.* Acta, Inc., Montreal, Canada. 822 pp, 1950.

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Obtaining Data by Telephone. A Clinical Evaluation of Hypnotic Drugs.

(22696)

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The widespread use of hypnotic drugs suggests that many people require some aid to fall asleep. Repeated studies at the Indianapolis General Hospital using the usual doses of various hypnotics have failed not only to demonstrate differences among hypnotic agents but also between the hypnotics and placebo. This paper presents our experience with telephone reporting.

Method. Tablets of Ethinamate (Valmid), Glutethimide (Doriden), Methyprylon (Noludar), and secobarbital sodium (Seconal Sodium) containing 500 mg, 500 mg, 200 mg, and 100 mg, respectively, were used. Envelopes containing one or 1½ tablets of these hypnotics or placebo were given to patients

TABLE I. Reported Time Lapse Prior to Onset of Sleep. Condensed form of data used in analysis. (Numbers in body of table refer to No. of reports in each category.)

Onset in min.	Tablets Placebo		Tablets Seconal		Tablets Valmid		Tablets Doriden		Tablets Noludar	
	I	II	I	II	I	II	I	II	I	II
0- 30	9	13	17	18	13	15	13	14	16	9
31- 60	9	4	6	5	5	6	7	12	7	7
61-120	5	7	3	2	9	6	4	1	2	6
121-480	4	3	1	2	0	0	3	0	2	5

receiving out-patient care. These envelopes were coded and directions as to order of administration given to the patients. The order of administration was also noted on the report form. The medications were given in a planned order so that randomization was obtained. Each morning the patients called the hospital to report the results of the previous night. Any of several persons answered the telephone and recorded the data. The questions were: The envelope number, the number of tablets taken, how soon they fell asleep, how long they slept, how well they rested, whether they were dizzy or drowsy when they awoke, whether they liked the medications and whether it helped them to sleep. Any other statements relating to the medication were also recorded. After the first 2 or 3 calls, some of the patients discontinued calling. They were called the same day to obtain their report and learn their reason for not calling. Following this show of interest on the part of the investigators, most of the patients did not fail again. Despite the many diversions which occurred, it was thought that adequate and accurate records had been obtained from 16 patients. These 16 patients completed the series, some several times, for a total of 27 complete reports on each medication at each dose.

Results. In the analysis of the data equal emphasis was placed on each of the 27 completed series of treatments. Since each series included the administration of each of 5 drugs at 2 doses, 270 daily reports were obtained. In plotting the curve for the onset of sleep (Table I) a skew distribution was observed. Transformation to logarithms, therefore, preceded variance analysis. Variance analysis indicates that sleep was significantly more prompt when hypnotics were used than when placebo was given. The differences among the drugs and between the 2 doses were not significant.

Analysis of the data on the duration of sleep (Table II) indicates significant heterogeneity among the hypnotics but placebo was not significantly different from the over-all hypnotic effect. This was an unexpected effect which on a clinical basis was illogical. Therefore, any comparison of the duration of sleep was not considered valid.

Analysis as to the number of times awakened, side-effects, and other data demonstrated no significant differences among the hypnotics, between the hypnotics and placebo, or between the two doses of drugs(1).

Discussion. By using adequate doses, differences among hypnotic drugs can be demonstrated in animals(2) and men(3,4). In these studies both the onset and duration of the central depression was measured. It was assumed that patients who had difficulty getting to sleep were capable of recognizing the effects of any drugs which enabled them to achieve the sleeping state more promptly.

TABLE II. Reported Durations of Sleep. Condensed form of data used in the analysis. (Numbers in body of table refer to No. of reports in each category.)

Duration in hr	Tablets Placebo		Tablets Seconal		Tablets Valmid		Tablets Doriden		Tablets Noludar	
	I	II	I	II	I	II	I	II	I	II
0 - 4	5	2	2	3	2	2	3	1	5	6
4.5- 6	10	5	5	4	11	8	4	7	2	8
6.5- 8	9	15	17	14	12	14	17	14	17	10
8.5-12	3	5	3	6	2	3	3	5	3	3

Patients were able to recognize a more rapid onset of sleep with hypnotics than with placebo. Unless the hypnotics were long acting and produced a "hangover" no after effects would be expected and none were noted.

Conclusions. 1. The onset of sleep is more prompt when usual therapeutic doses of hypnotics are administered to patients. 2. Duration of sleep and incidence of undesirable effects produced by Seconal, Valmid, Doriden, and Noludar were not significantly different from those produced by placebo.

We wish to thank Miss Toledo Syster, R.N., Dr. A. S. Ridolpho and Dr. Robert E. Johnston for helping in this project.

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1. Cochran, W. G., and Cox, G. M., *Experimental Designs*, John Wiley and Sons, Inc., New York, 1950.
2. Gruber, C. M., *J.A.M.A.*, 1941, v117, 1147.
3. Gruber, Jr., C. M., Kohlstaedt, K. G., Moore, R. B., and Peck, Jr., F. B., *J. Pharm. and Exp. Therap.*, 1954, v112, 480.
4. Foltz, E. L., Dracos, F., and Gruber, Jr., C. M., *Am. J. Med. Sci.*, 1955, v230, 528.

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Character of Agent Inducing Leukemia in Newborn Mice.* (22697)

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It is now well established that there are filterable agents capable of causing leukemia or tumors, or both, in several species of animals, and Stanley(1) concluded that many, if not all, tumors in man as well as in animals could be caused by viruses. Of current interest is the virus‡(2) in tissues of high leukemia strains of mice that will induce leukemia when injected into mice of low-leukemia strains less than 1 day old. Technical difficulties have greatly handicapped confirmation and research on the nature of this agent: the necessity of using animals less than 12 hours of age; the extraordinary host specificity; the long latency period (about 7-12 months) which is close to that of spontaneous leukemias; the variable and usually small percentage of leukemias induced; the necessity of using large numbers of controls to ascertain that the tumors were induced and not spontaneous; and the performance of bioassays to further exclude contamination of the inoculum with living cells.

Two sets of experiments are reported here. The first was initiated with Dr. Ludwik Gross. Motivated by the thought that if a virus causes leukemia it may play a determining role in leukemia induction by radiation, Dr. Gross was invited to Oak Ridge in July, 1953, to demonstrate his technic. The second set of experiments were set up in Boston primarily to establish the sensitivity of different low-leukemia strains to the agent in Ak tissue. The immunogenetic character of many leukemias was tested by transplantation assays, since one or a few immature or leukemic cells from Ak donors may have been present in the inoculum and may have survived when grafted in newborn mice, giving rise to leukemia close to the "leukemia age." It was assumed that virus-induced leukemias have the immunogenetic character of the recipient strain, and cell-induced leukemias that of the donor strain.

Materials and methods. Strains of mice. Low-leukemia strains, C3H (abbr. H), LAF_1, DBA/1, BALB/c, Rf, and C57BR, were used. The tissue extracts containing the agent were prepared from Ak leukemic mice. Sublines of Ak and H mice are indicated as follows: a = Andervont, g = Gross, r = Russell, b = Bittner, R = Rockefeller Institute (Lynch), o = Oak Ridge, eb = mam-

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‡ The terms virus and agent are used interchangeably for the substance in tissue extracts capable of inducing or hastening the development of leukemia.

TABLE I. Results of Injections of Ak Leukemia Extracts into Newborn Mice.

Donor		Recipients					
						Total	
Strain	Material	Ha	Hg	Hr	Hb/Hr	No.	%
Ak/g	Centrifugate	2/5*	—	3/25	—	5/ 30	16.7
"	Filtrate	4/16	3/4	1/34	2/11	10/ 65	15.4
Ak/o	"	1/2	—	1/17	5/19	7/ 38	18.4
AkR	Centrifugate	—	—	4/6	—	4/ 6	66.7
	Total No.	7/23	3/4	9/82	7/30	26/139	
	%	34.8	75.0	11.0	23.3		18.6
	Control No.	1/10	0/15	4/45	7/34	12/104	
	%	10	0	8.8	20.6		6.7

* No. positives (leukemias) over total No. of mice inj.

mary agent-free line of the Roscoe B. Jackson Memorial Laboratory. The use of the indicated sublines of C3H and Ak as both source of the agent and host was by necessity. *Preparation of extracts.* The technic of Gross was slightly modified in the second experiment. Extracts of leukemic tissue were prepared from a recently isolated strain of transplantable AkR leukemia. Spleen, lymph nodes (not including cervical nodes in the second experiment because of their proximity to the salivary gland which may be carrier of another virus), thymus, and a small piece of liver were ground in a mortar with enough cold normal saline to make a 20% suspension, centrifuged at 3000 rpm for 10-15 minutes in a refrigerated centrifuge set at 0°C, and the supernatant fluid drawn off. The supernatant fluid was recentrifuged at 0°C at 3000 rpm for 15-30 minutes or 7000 g for 10 minutes, and the supernatant fluid again drawn off. In the second experiment, the floating fat was removed with a sterile swab. The extract was kept on ice in the refrigerator and used within 24-30 hours after preparation. Filtration was omitted in the second set of experiments in order to avoid further weakening of the extracts and because emphasis was placed on the character of induced leukemic cells. Instruments and equipment were chilled. *Inoculations.* Babies less than 12 hours old, and some 12-24 hours old were injected subcutaneously in the back with 0.05-0.1 cc of inoculum through a 27-gauge needle. In some instances, supplementary intraperitoneal injections were made to make a total dose of 0.1 cc. Implants of leukemic cell suspension in adults were made intramuscularly in the thigh. *Termination of experiments.* In the first experiment, nearly all mice were observed until natural death. The second experiment was terminated when the last surviving animals were 10-15 months of age (about 38% of the total number being younger than one year). This seemed permissible because of our limited objectives, the spontaneous leukemias in question being predominantly thymic and appearing mostly under 10 months of age.

Results. First experiment. The results of the first set of injections of Ak leukemia extracts into newborn mice (all less than 12 hours old) are summarized in Table I. In one subline (Hb/Hr), the incidence of leukemia among the controls was almost as high as in the experimental group and higher than the average in the various H sublines used. In subline Hr the difference between experimental and control groups was also slight. Transplantation assays were made on available mice in ignorance of the marked subline differences among C3H (=H) mice (Table II). On first glance, the results appear to

TABLE II. Transplantation Assays of 4 Leukemias Developing in Agent Injected Mice.

Donor			Recipient*		
No.	Strain	Material	AkR	Ak/o	Hr
Hb/Hr 26	Ak/o	Filtrate		0/ 6	0/11
10	Ak/g	"		0/ 4	1/ 5
Hr 47	AkR	Centrifugate	0/20		12/12
45	"	"		0/ 4	1/ 6
		Total	0/20	0/14	14/34

* Each recipient received intraper. 0.1 ml of a leukemic cell suspension, and was observed until natural death or for a period of at least 3 mo after inoculation.

TABLE III. Induction of Leukemia in Newborn Mice with AkR Leukemic Tissue Extract.

			Leukemia									
					Months postinjection							
Strain	Material†	No. survivors 4 mo*	No.	%	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–15
LAF_1	E	53	0									
	S	66	0									
Heb	E	54	8	15	3	1		1	2		1	
	S	42	0									
DBA/1	E	47	9	19		1	1	1	1	1		4
	S	35	0									
BALB/c	E	33	1	3			1					
	S	33	0									
Rf	E	29	10	38	1	2	2	2	1		2	1
	S	43	0									
C57BR	E	55	1	2							1	
	S	53	0									
AkR	E	34	26	76	2	3	5	6	3	3	3	1
	S	21	10	48			1	1	1	4	2	1

* Discounting from total a few mice on which adequate autopsy could not be performed.
† E = Extract; S = Saline.

confirm the findings of Gross in that 3 of 4 leukemias could be grafted on H mice, none on Ak mice, and one on neither strain. However, only one (Hr47) had a distinctly H (Hr) type of leukemia, but in this subline the difference in incidence of leukemia between agent-injected and control mice was negligible; hence the disease might have been spontaneous. The two leukemias arising in the Hb/Hr subline, which one would expect to be transplantable to all Hr mice, took in only 1 of 16 animals. Thus these findings cannot be taken as evidence that these leukemias were of the recipient immunogenetic types and hence induced by a virus. Interpretation of these results calls for ascertaining the degree of homozygosity and the immunogenetic relationship of the various sublines.

Second experiment. Leukemia induction. It seemed desirable to search for a low-leukemia strain with high sensitivity to the Ak agent, preferably a strain with less immunogenetic relationship with Ak as is known to exist between some sublines of C3H and Ak (3,4). Accordingly, newborn mice of 7 strains were injected with tissue extracts from mice with AkR leukemias (Table III). The extracts were presumably cell-free, and transplantation assays (Table V) suggest that most induced leukemias were certainly not induced by contaminating cells.

Four strains of animals proved fairly sensitive to induction of leukemia with Ak agent: Heb, DBA/1, Rf, and the high-leukemia strain, AkR, from which the agent was obtained (Table III). Leukemia was induced in 15% of a breast tumor agent-free subline of C3H (Heb) mice; none occurred among the controls. (Sublines of greater sensitivity were not available.) Leukemia was induced in 19% of DBA/1 mice; none occurred among the controls.

Of the low-leukemia strains, Rf proved most susceptible; 38% of agent-injected mice developed leukemia, at a time when none of the controls had it. Originally, the Rf strain had only 2-3% spontaneous leukemias, and it had no genetic relation to the Ak strain(3). In recent years, a small percentage of Rf mice "take" Ak leukemic cells. The spon-

TABLE IV. Transplantation Range of AkR Leukemic Cells.

	Leukemia	
Recipient strain	Adults	<1 day old
LAF_1	0/ 11	4/ 5
Heb	0/ 27	
DBA/1	0/ 7	
BALB/c	0/ 11	
Rf	17/174	
C57BR	0/ 8	12/13
AkR	18/ 19	10/10

One spontaneous leukemia in an Rf mouse was grafted on Rf and AkR mice with results as follows: Rf hosts 6/6, AkR hosts 0/5.

TABLE V. Transplantation Assay of Leukemias of Mice Injected with AkR Extracts.

Leukemia donor		Original passage				Subpassage I				Subpassage II			
		In strain of origin		In AkR		In strain of origin		In AkR		In strain of origin		In AkR	
No.	Age†	Result	Latency‡	Result	Latency	Result	Latency	Result	Latency	Result	Latency	Result	Latency
Heb 17	141	*2/6	55, 93	1/6	97	7/11	29–37						
						3/ 6	36	*1/6	83	0/5		5/5	44-48
20	141	*4/4	21–45	1/6	84	17/17	16–21	*6/6	23–28	4/4	15–37	4/4	14 15
						19/19§	19–20						
16	251	0/5		2/5	60, 205								
Rf 55	163	0/3		4/5	92–183								
56	163	3/5	16–18	1/5	69								
57	262	5/5	18–60	2/6	59, 108								
7	217	*4/6	27–42			1/ 4	44	0/4					
				*3/6	83–100			3/3	29–33				
12	143	2/6	136, 159	1/5	137								
16	237	0/6		0/5									
DBA/1 1	435	2/5	53, 82	0/3									
8	428	1/5	74	0/4									
9	428	*5/5	45–49	0/4		5/ 5	18–20	0/5					
BALB/c 13	186	*9/9	17–19	0/8		1/ 3	48	0/4					

* Indicates group from which subpassage was made. † In days, when mouse was sacrificed for transplantation. ‡ No. of days after graft. All AkR recipients were $1\frac{1}{2}$-$2\frac{1}{2}$ mo of age when grafted. § Frozen cells from same donor as transfer above.

TABLE VI. Mortality Data on Newborn Mice Injected with Leukemia Extracts (Second Experiment).

						Mortality					Total mortality, 4-10 mo					
						1–4 mo		4–10 mo			Leukemia		Other causes		Combined	
Strain	Material	No. inj.	No. weaned	% loss	No. survivors 4 mo	No.	%	4–6	6–8	8 10	No.	%	No.	%	No.	%
LAF₁	E*	87	59	32	54	5	8†	1	0	0	0		1	2‡	1	2‡
	S*	110	66	40	66	0		0	0	0	0		0		0	
Heb	E	112	62	45	55	7	11	9	9	6	7	13‡	17	31	24	44
	S	100	51	49	43	8	16	2	2	0	0		4	9	4	9
DBA/1	E	155	55	65	50	5	9	4	7	6	5	10	12	24	17	34
	S	138	38	71	38	0		5	0	3	0		8	21	8	21
BALB/c	E	54	39	28	34	6	13	2	6	1	1	3	8	23	9	26
	S	52	39	25	33	6	15	1	3	2	0		6	18	6	18
Rf	E	148	42	72	29	13	31	4	6	2	8	27	4	14	12	41
	S	112	49	56	43	6	12	0	0	0	0		0		0	
C57BR	E	82	60	27	55	5	8	2	1	1	0		4	7	4	7
	S	77	55	29	53	2	4	4	1	2	0		7	13	7	13
AkR	E	103	63	61	36	4	10	6	12	6	22	61	2	6	24	67
	S	103	77	75	22	4	15	2	3	5	7	32	3	14	10	46

* E = Extract; S = Saline. † % of those weaned. ‡ % of those surviving 4 mo.

taneous incidence of leukemia remained low in the Rf strain, the unique feature of which is an extraordinary susceptibility to the induction of myeloid leukemia by ionizing irradiation(5,6).

Injection of newborn AkR mice with AkR extract greatly shortened the latency period of leukemia and increased the leukemia incidence as reported recently by Rudall *et al.* (7).

Immunogenetic Relationships. Table IV shows that adult Rf mice are sensitive to AkR cells, but "takes" occur in only about 10% of them when injected with large numbers of Ak leukemic cells. Adult mice of the other 5 strains proved resistant to AkR cells. Newborn mice of 2 strains (LAF_1 and C57BR), which proved resistant to the agent, were receptive to AkR cells. This indicates that histoincompatibility forces do not function in newborn mice.

Transplantation assays for the immunogenetic character of 13 leukemias developing in agent-injected mice (3 in Heb, 6 in Rf, 3 in DBA/1, and 1 in BALB/c) are summarized in Table V. All leukemias were grafted on mice of the strain of origin and on AkR mice. In 5 cases, further subpassages were made.

In the Heb strain, the 3 leukemic mice were 141-251 days old when sacrificed for transplantation assay. Two had marked generalized leukemia with thymic involvement, and proved to be immunogenetically related to the AkR strain. The grafted leukemias grew faster and in greater percentage in the strain of origin than in AkR. However, when a subpassage was made from the first-generation graft in an AkR mouse from Heb 17, leukemia developed in all of 5 AkR and in none of the strain of origin (Table V). It is unlikely that the leukemia was spontaneous in this donor AkR mouse because the latter was only 4 months old when sacrificed. The leukemia in Heb 20 certainly appeared "bivalent," since it "took" in all injected AkR and Heb mice. The third, presumably agent-induced leukemia (Heb 16), was microscopically an early case. Transplantation failed in the strain of origin but apparently succeeded in 2 AkR mice. One of these was probably transplanted, since it developed in a 4-month-old mouse (60 days after the graft) and was nonthymic. The second, being thymic and occurring late, may have been spontaneous.

Of the 6 transplanted leukemias arising in Rf mice, one grew in AkR only, 4 grew in both Rf and Ak, and one in neither strain. The one which appeared to be of the AkR type (Rf 55) could have been derived from Ak cells contaminating the extract. If so, it was latent in the Rf host for about 163 days. In general, the leukemias that were "bivalent" grew faster and in greater percentage in Rf than in AkR mice. Again, a subpassage from an AkR leukemia grew well in AkR. It is possible that this was spontaneous, since the donor mouse was nearly 5 months old when this subpassage was made. All 4 Rf mice grafted from this AkR died of infection 17 days after the graft, and had to be discounted. The 3 DBA/1 and 1 BALB/c leukemias grew only in the strain of origin. The very long induction period (428-435 days) of the 3 DBA/1 leukemias raises the question whether these cases were not spontaneous.

These data point to the necessity of carrying out transplantation assays on a much larger scale and with the use of proven highly inbred strains of mice with good genetic markers.

Mortality in agent-injected mice. Duran-Reynals has shown(8) that an oncogenic virus can cause a non-neoplastic disease in newborn animals. Mortality data (Table VI) were gathered to answer the question whether or not diseases other than leukemia might have been caused by the agent and whether some leukemias might have been missed at autopsy.

Table VI indicates that, in general, infant mortality was equally high among agent- and saline-injected animals. Indeed, in 5 strains mortality was slightly higher among the controls and in only 1 (Rf) was it distinctly higher among the extract-injected animals.

Leukemias did not occur until after the mice were 4 months old. In the interval between weaning and 4 months of age, mortality was higher among agent-injected mice in 4 strains, but in only 2 of these (DBA/1 and Rf) was there a definite increase of leukemia incidence in subsequent months. In 2 strains in which leukemia development was hastened and/or increased (AkR and Heb) mortality was higher among the saline-injected animals, even during the first 4 months. Mortality was caused by common infections and parasitisms.

In the 4 low-leukemia strains in which leukemia developed in extract-injected animals and was absent in saline-injected animals during the first 10 months of life, mortality from causes other than leukemia was invariably higher among the agent-injected animals, the difference being greatest in the 2 strains in which the incidence of leukemia was the highest (Rf and Heb). Since no systematic microscopic examination was made in animals grossly free of leukemia, it is not possible to state whether all those mice recorded to have died from causes other than leukemia were actually free from this disease, or whether the presence of the agent had lowered resistance to common infections, or otherwise caused death. The total mortality between the ages of 4 and 10 months appears to be as good an index of viral activity as the presence of grossly detectable leukemia.

Salivary gland and other tumors. Gross suggested that the virus carried in Ak mice is multipotent, capable of producing salivary gland and other tumors, or that Ak mice carry several oncogenic viruses. At autopsy, careful search failed to reveal neoplasms other than leukemias, except for two questionable salivary gland tumors in the first series. These findings do not support the view that the salivary gland tumors are caused by the same agent that induces leukemia.

Three salivary gland tumor strains (C3H) received from Dr. Gross were transplantable in C3H mice with variable success; none took in 100% of the animals and some regressed. They were not transplantable in Ak mice. This is in line with the work of others(9,10), indicating poor transplantability of these parotid tumors. The parotid glands of many C3H mice carrying grafted salivary gland tumors were examined microscopically. In none were incipient tumors noted, nor was there any evidence of other tumors or leukemias in hosts bearing such grafts.

Discussion. Three processes will now be considered by which leukemia can be caused: transplantation, induction, and transduction. Cellular grafts from spontaneous leukemias retain, as a rule, the genetic character of donor cells, whereas grafts from induced leukemias resemble closely or are identical with those of the host cells. Although this is the rule, it has also been reported that methylcholanthrene-produced leukemias differ among themselves immunogenetically(11). Bioassays indicate that leukemic cells are not present in young animals with genetic liability to leukemia(12). A primitive hemopoietic cell may become established in homologous newborn mice of diverse strains, and undergo leukemic transformation after a latency period (close to that of spontaneous leukemia). In this case, the leukemic cells are also expected to be of the donor type, and the process would be that of *transplantation* of precursor cells. Gross(13), and Woolley(14) working closely with Gross, have stated that their virus-induced leukemias were of the recipient type; this would indicate *induction*. Analysis of our transplantation results points to difficulties of interpretation, and suggests the possibility that some induced leukemias have the genetic character of both donor and recipient. Such a finding would be best explained by supposing that the transmitting agent is related to chromosomal matter (probably nucleoprotein) of the leukemic cells or their precursors which, like the DNA of certain microorganisms, can enter the reproductive matter (DNA genome) of other "infected" cells, either multiplying with them or causing perpetuation of their special genic characteristic by some other mechanism. Lederberg(15) named such processes *transduction*. Although it is doubtful that the transformations induced with lysogenic viruses (bacteriophage) are analogous with that of induced transformation of pneumococci, the term of Lederberg is accepted as a matter of convenience. In contrast, common infectious viruses of mammals are best conceived as fully perpetuating themselves, the host merely contributing a biochemical environment, favorable to their multiplication.

The experiments here reported are inadequate to arrive at a definite conclusion on the nature of the agent which induces or hastens the development of leukemia in mice. Information is wanted on the effects of injection of extracts from low-leukemia strain mice,§ characterization of agents after passage through different strains, better correlation of immunologic and genetic relationships, and attempted inactivation of the agent with DNA-ase. Simple technical difficulties as encountered here in interpreting findings with this type of experiment will have to be overcome. How to certify inocula to be free from cells, and strains for adequate homozygosity? How long is the latency period of leukemias caused by transplantation of one or a few leukemic cells shortly after their origin? It is well known that neoplastic cells rapidly gain growth vigor in the course of passages, but it is not known when the neoplastic transformation occurs and how rapidly leukemic cells multiply in the original host. Inadequate inbreeding of the mouse strains, or lack of complete autonomy or genetic difference between strain and induced neoplastic cells could possibly account for non- or poor transplantability of some leukemias in the primary transfers. The strains used by us were adequately inbred for uniform success in transplantation of well-established leukemias, but inbreeding is a relative matter. Subtle immunogenetic changes are conceivable even in the course of neoplastic transformation in spontaneous leukemias, and antigenic simplification is a common occurrence in the course of transplantation.

A major problem requiring clarification is the type of leukemia induced in relation to the source of the agent. In the Ak strain, most spontaneous leukemias (80-90%) are thymic in origin. Is the thymus the sole or most sensitive organ attacked by this agent? In the present study all leukemias in agent-injected Ak mice were lymphoid with thymic involvement. In the other strains, however, many were generalized with involuted thymuses. Typing of leukemias requires special efforts not done systematically in the present study. The morphologic problems to be resolved include those of differentiation of advanced myeloid leukemoid reaction from leukemias, and of granulomatous lesions from neoplasms of monocytes (reticulum cells). Such a study calls for correlation of morphology with bioassays for malignant (autonomous) cells. Spontaneous leukemias in Ak mice are preventable by thymectomy(3). It follows that leukemia induction by this Ak agent would also be preventable by thymectomy. Attempts to recover the agent from nonthymic, spontaneous, and variously induced leukemias, and determination of the range of agents from different sources will have to be done. Since most nonthymic leukemias appear late in life, lifespan studies will be unavoidable. The Rf strain appears well suited for such studies because of its broader spectrum of sensitivity. In this strain, ionizing radiation causes thymic lymphomas (preventable by thymectomy)(5), myeloid (not so preventable), and reticulum-celled (monocytic) leukemias.

Three processes have been mentioned that could explain the development of leukemias following injection of tissue extracts from high-leukemia strain mice: transplantation, induction, and transduction. The first can now be excluded as a mere occasional complicating factor, the latter two are possibilities that merit equal consideration. Much has been said by others in favor of induction; the present work underlines the theory of transformation (transduction). Experiments suggesting it have been described and contradicted(3,16), and those here reported are likewise not conclusive. But the theory of transduction based on experiments on pneumococcal transformation would explain best the observed facts: the necessity of using newborn mice; the reported high sensitivity of strains which have some genetic relation to the Ak strain; the non-infectiousness of this agent under natural conditions; and the very long latency period, which is about that of the spontaneous disease. Latarjet(17) considered the possibility of the agent being similar to a prophage, but his experiments argue against this.

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§ Latarjet (personal communication) failed to demonstrate an agent in his low-leukemia line 17.

In general, causation of leukemia by virus can be conceived as: a) Stimulation of normal cells by intracellular specific or nonspecific "parasites." This would imply that the leukemias are virus-conditioned neoplasms. b) Induction of somatic mutation by a resident virus, proposed by Murphy(18) who named avian tumor viruses "mutagens." Mutation and abnormal differentiation, another possibility, imply an irreversible induction change in the cells, mutation being genetic and differentiation nongenetic in character. c) Recombination of chromosomes as occurs with transduction of genetic features of pneumococci and other microorganisms. Presently, all these possibilities(16) are useful as "working hypotheses."

Summary. 1. Tissue extracts from mice of the high-leukemia strain Ak injected into newborn mice greatly increased the leukemia incidence in Rf, DBA/l and C_3Heb mice, and greatly hastened the development of leukemia in AkR mice. 2. Mortality without gross anatomic manifestation of leukemia was also enhanced in extract-injected mice of low-leukemia strains. 3. Although all leukemias in Ak mice were lymphoid with thymic involvement, many leukemias in the low-leukemia strains were of non-thymic types. 4. Transplantation assays of leukemias induced in the low-leukemia strains disclosed a heterogeneity in types, some being related immunogenetically with both host and donor strain. 5. Neither salivary gland nor other tumors were induced by the leukemia agent studied. 6. The available information suggests consideration of a process analogous with DNA-induced transformation (transduction) of genetic properties of microorganisms rather than to a natural type of viral infection.

We gratefully acknowledge the cooperation of Dr. Ludwik Gross in demonstrating his technics at Oak Ridge (first experiment), Drs. William Russell, H. B. Andervont, John J. Bittner and Ludwik Gross for mice of special sublines, and Mrs. Peggy Ledford and Miss Esther-Mary Farrington for devoted technical assistance.

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1. Stanley, W. M., *Proc. Third Nat. Cancer Conf. (Detroit)*, 1956.

2. Gross, L., *Leukemia Research (Ciba Foundation Symposium)*, Little, Brown and Co., Boston, 1954, p76.

3. Furth, J., *Blood,* 1951, v6, 964; and references cited.

4. ———, *Leukemia Research (Ciba Foundation Symposium)*, Little, Brown and Co., Boston, 1954, p97.

5. Upton, A. C., and Furth, J., *Proc. Third Nat. Cancer Conf. (Detroit)*, 1956.

6. ———, *Blood,* 1954, v9, 686.

7. Rudall, G., Duplan, J. F., and Latarjet, R., *Compt. rend. Acad. d. sc.*, 1956, v242, 837.

8. Duran-Reynals, F., *Am. J. Med.*, 1950, v8, 490; and references cited.

9. Stewart, S. E., *J. Nat. Cancer Inst.*, 1955, v15, 1391.

10. Law, L. W., Dunn, T. B., and Boyle, P. J., *ibid.*, 1955, v16, 495.

11. Furth, J., Boon, M. C., and Kaliss, N., *Cancer Research,* 1944, v4, 1.

12. Furth, J., and Boon, M. C., *AAAS Res. Conf. on Cancer (Washington, D.C.)*, 1945, 129.

13. Gross, L., *Cancer,* 1956, v9, 778.

14. Woolley, G. W., *Proc. Third Nat. Cancer Conf. (Detroit)*, 1956.

15. Lederberg, J., *Am. Scientist*, 1956, v44, 264.

16. Hauschka, T. S., and Furth, J., in *The Leukemias: Etiology and Pathophysiology (Proc. Internat. Symp., Detroit)*, 1956.

17. Latarjet, R., *Proc. Sixth Internat. Congr. Internat. Soc. of Hematology (Boston)*, 1956.

18. Murphy, J. B., *Tr. A. Am. Physicians,* 1931, v46, 182.

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Oxygen Consumption in the Rat During Prolonged, Acute Starvation.
(22698)

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The oxygen consumption of animals(1,2) and humans(1,2,3) is known to be depressed by starvation. There is also ample evidence that atrophic changes occur in the thyroid gland(1,4) and that histological and histochemical alterations appear in the anterior pituitary gland during inanition(1,4). These changes are assumed to be the explanation for the depression in oxygen consumption during starvation. It is, however, entirely probable that such changes are secondary to alterations of peripheral cellular metabolism.

The latter hypothesis was tested in hypothyroid, euthyroid and hyperthyroid rats subjected to prolonged, acute starvation.

Methods and materials. All animals were young, mature female rats of the Holtzman Farm weighing between 200-250 g. Thyroidectomy was performed under sodium pentabarbital (nembutal, 30 mg/kg I.P.). The soft tissues of the necks of random experimental animals were removed *en bloc*, sectioned serially and examined histologically for evaluation of completeness of surgical ablation of the gland. Animals were used 5 to 6 weeks after thyroidectomy. Hyperthyroid animals were prepared by subcutaneous, daily injection of 10 γ of 3-5-3′ triiodothyronine.* Injections were begun 3 days prior to the commencement of starvation. Starvation was instituted by removal of food from the cages at 9:00 a. m. of the day on which the experiment was begun. At noon, the rats were weighed on a triple beam balance to the nearest 0.5 g, which was taken as the initial weight. Animals were weighed approximately the same time each day thereafter. After removal of food (Purina Laboratory Chow) the animals received water *ad libitum* but no food. All animals were housed in individual cages and kept in an airconditioned room (about 75-80°F.) Oxygen consumption was measured by a method described by Grad(5). At least three 15-minute determinations were averaged to establish each oxygen consumption point reported. The formula used to express the oxygen consumption is: Liters/m²/hr $= \frac{11.2 \text{ x } (\text{wt}^{2/3})}{100}$ where weight is in kg and the figures corrected to standard temperature only.

TABLE I. Oxygen Consumption in the Normal, Thyroidectomized and Triiodothyronine Treated Rat.

Treatment	No. of animals	O_2*	S.D.†
Normal	52	6.63	.43
Thyroidectomized	6	5.20	.51
Triiodothyronine	11	8.10	.86

* O_2 = Oxygen consumption in liters/m²/hr.
† S.D. = Stand. dev. of mean.
Triiodothyronine was given for 3 days (10 γ/day subcut.) and oxygen consumption measured on 4th day. Animals were without food for 4 hr.

Results. Determinations of oxygen consumption in a series of 52 normal animals resulted in a mean value of 6.63 ± 0.43 liter/m²/hr. A comparison of the oxygen consumption of normal, thyroidectomized and triiodothyronine treated rats on day zero (starting day of starvation) is shown in Table I.

Fig. 1 shows the daily oxygen consumption course of the starving rats in each treatment group. Because of space and time limitations it was impossible to measure each animal's oxygen consumption daily. Table II indicates number of animals used in determining each point of the graph. There was no selection of animals to be tested on any given day. No observation has been deleted from this report. The original size of the groups in the experiment was: 11 intact, 11 triiodothyronine treated animals and 12 thyroidectomized animals.

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* We are indebted to Dr. T. A. Schwab of Smith, Kline and French Laboratories for a generous supply of triiodothyronine.

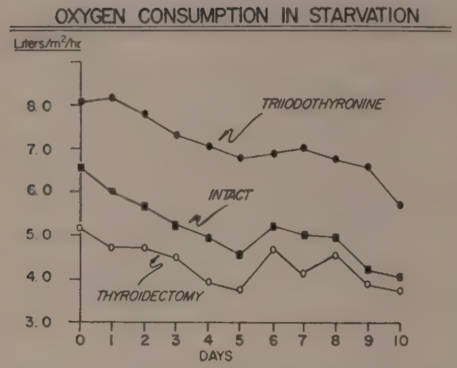

FIG. 1. Oxygen consumption during starvation of female rats. Triiodothyronine was given subcut. each day in the amount of 10 μg and was started 3 days prior to the commencement of starvation. Thyroidectomized animals were prepared surgically and allowed 5-6 wk to develop hypothyroidism.

It will be noted that there is a decrease in oxygen consumption in all 3 groups subjected to progressive starvation. The decrement seems absolutely greater in the normal than in the triiodothyronine treated or in the thyroidectomized groups. The thyroidectomized group shows the least decline in oxygen consumption during acute starvation. In the triiodothyronine treated and thyroidectomized groups there seems to be a relative plateau reached at about the 4th day and maintained until about the 9th day. The triiodothyronine group showed a considerable mortality by this time (commencing on the 6th day of starvation), and the experiment was terminated on the 10th day of starvation (which is about 0.5 day longer than the mean survival time for intact starving rats in our laboratory; range 9 to 11.5 days). The thyroidectomized animals live 2 to 2½ times as long as the intact, starving animals.

There seems to be a marked similarity between the triiodothyronine treated and thyroidectomized curves, while the curve of the intact starving rats deviates somewhat from their general configuration by a more sustained and steeper oxygen consumption fall.

TABLE II. Number of Animals Determining Each Point on Graphs of Fig. 1.

Treatment	Day 0	1	2	3	4	5	6	7	8	9	10
Triiodothyronine	11	10	9	10	10	2	2	8	8	7	2
Intact	52	10	5	9	8	2	3	5	8	4	3
Thyroidectomized	6	5	8	3	5	2	1	5	5	3	3

Discussion. It is demonstrated that starvation is capable of reducing the oxygen consumption of the female rat independently of thyroid activity. This decrement in oxygen consumption is apparent in rats in which the thyroid gland is absent, as well as in animals rendered hyperthyroid by administration of 10 gamma of triiodothyronine per day.

It should be pointed out that administration of a constant daily amount of triiodothyronine to the starving rat amounts to an increasing daily dose when calculated on the basis of dose to body weight.

Whether this depression in oxygen consumption is due to loss of specific dynamic action of food, lack of sufficient available substrate at the cellular level, or other causes, can not be answered by the present experiment. Investigation of these various possibilities is in process.

Summary. Hypothyroid, hyperthyroid and euthyroid rats were subjected to acute starvation to extremis. Their oxygen consumption was measured daily. All groups showed an approximately equivalent early depression of oxygen consumption which is independent of the absolute level of the initial oxygen consumption.

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1. Keys, A., Brozek, J., Henschel, A., Mickelsen, O., and Taylor, H. L., *Human Starvation*, Univ. of Minnesota Press, Minneapolis, Minn., 1950.

2. Quimby, F. H., Phillips, N. E., and White, I. U., *Am. J. Physiol.*, 1948, v154, 188.

3. Morgulis, S., *Fasting and Undernutrition*, Dulton and Co., New York, 1923.

4. Mullinos, M. Q., and Pomerantz, L., *J. Nutrition*, 1940, v19, 493.

5. Grad, B., *Endocrinology*, 1952, v50, 94.

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Effects of *trans* Fatty Acid Isomers upon Essential Fatty Acid Deficiency in Rats.* (22699)

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Recently it has been found that diets containing hydrogenated fats cause degeneration of spermatogenic tissue in weanling rats(1). Hydrogenated fats contain considerable proportions of *trans* isomers of the unsaturated fatty acids(2). Therefore it became of interest to learn if known *trans* isomers of the unsaturated acids have any effect upon spermatogenic tissue and if they are deposited in the animals. The most obvious unnatural fatty acid isomers to test for this effect are the *cis, trans* and *trans, trans* isomers of linoleic acid. These acids have been demonstrated to possess no essential fatty acid activity(3,4) and they are not oxidized by lipoxidase. Moreover, the all *trans* isomer of linolenic acid has been found to act as an effective competitive inhibitor of lipoxidase oxidation of linoleate(5), and it might be expected that the *trans* isomers act as competitive inhibitors of enzyme reactions involving essential fatty acids. Elaidic acid might also be suspected of antagonism to the essential fatty acids by the same reasoning. Therefore it was decided to test these substances for their overall effects on essential fatty acid (EFA) deficiency and upon the development of spermatogenic tissue. The quantities of these acids available did not allow a detailed or full investigation of their effects, but some effects have been observed in a few animals.

Methods. Male 15-day-old rats were fed a depletion diet containing 18% casein, 67% sucrose, 4% Wesson salts, 4% celluflour, 1% hydrogenated coconut oil, 1% cholesterol, 2% sulfasuxidine, and 3% urea plus required vitamins. The inclusion of cholesterol has been demonstrated to accelerate EFA deficiency(6). At the end of 3.5 months, all rats had developed severe EFA deficiency symptoms. The rats were then divided into pairs and were given a diet containing 18% casein, 73% sucrose, 4% salts, 4% celluflour, and 1% hydrogenated coconut oil plus the required vitamins. The fatty acid esters were then administered as oral supplements at the rate of 0.1 ml per day for 3 weeks. The rats were weighed and scored biweekly during the period of supplementation. At the end of this period, they were killed, and the left testis was removed from each for histological examination. The carcasses were extracted with boiling ethanol and petroleum ether. The lipid was saponified, the unsaponifiable matter was removed, and the free fatty acids recovered. The fatty acids from the odd-numbered rats were separated into "saturated" and "unsaturated" acids by crystallization from 10 volumes of petroleum ether per weight of acids at —40°. Fatty acids from even numbered rats were esterified with methanol for use in determining the content of *trans* unsaturation. The values are expressed as elaidic acid. Ethyl elaidate (*trans*-9-octadecenoic acid ethyl ester) was prepared from elaidic acid (m.p. 44°). Ultraviolet absorption was $k_{268} = 0.025$ and $k_{233} = 0.18$. Ethyl linolelaidate (*trans*-9, *trans*-12-octadecadienoic acid ethyl ester) was prepared by the method of Kass and Burr(7). Infrared spectrum showed no *cis* double bonds to be present. Ultraviolet absorption indicated the presence of about 7% conjugated diene acids. $k_{268} = 0.18$, $k_{233} = 6.7$. Methyl *cis*-9, *trans*-12-linoleate was prepared by Dr. O. S. Privett. The preparation had strong absorption bands at 10.28 μ (*trans*) and 3.3 μ (*cis*). $k_{233} = 0.52$ and $k_{268} = 0.19$. Ethyl linoleate (nat. *cis*-9, *cis*-12-octadecadienoic acid ester) was prepared by low temperature crystallization of safflower oil fatty acids. Infrared spectrum showed evidence of a trace of *trans*

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TABLE I. Effects of Fatty Acid Supplements upon E.F.A.-Deficient Rats.

Rat No.	Supplement	Wt gain, g	Score change*	Testis /body wt, %	Spermatogenic tissue†	Carcass fatty acids, g	*Trans* acids, %‡	Total *trans* acids, g‡	Gain/g *trans* acid‡
1	Ethyl elaidate . . .	48	2.0		1	12.8			
2		40	– .5		2	12.2	1.4	.17	258
3	" linolelaidate	27	2.0	.13	1	20.8			
4		10	2.0	.22	1	13.5	2.7	.36	50
5	Linolelaidate + linseed oil	71	0	.28	3	18+			
6		74	–1.5	.30	3	29.6	3.9	1.15	63
7	*cis, trans* linoleate	21	2.0	.34	3	12.6			
8		9	3.0	.20	2	9.8	3.0	.29	51
9	*Idem* + linseed oil	51	– .5	.26	4	32.3			
10		63	–2.0	.18	2	25.5	2.3	.59	97
11	Linolenate	42	– .5	.27	2	28.1			
12		92	1.5	.29	2	28.7	.9	.26	259
13	Linoleate	56	–2.5	.24	2	27.1			
14		50	–4.5	.31	4	33.5	1.2	.40	132
15	Linseed oil	62	–2	.35	4	22.7			
16		61	1.5	.28	4	29.3	0	0	0
17	None (fat-free)	31	1.0	.29	2	11.5			
18		36	.5	.22	1	19.0	0	0	0
19	Deficient initial controls					4.0			
20						6.1	1.4	.1	

* Scores of 0-3 for scaliness of feet, 0-4 for scaliness of tail, and 0-2 for roughness of hair coat were added.

† Degree of recovery was graded from 0 to 5 as follows: Degree 0: Tubules appear atrophic and contain only syncytium of Sertoli cells with few scattered spermatogonia. 1: Spermatogonia and spermatocytes present, but epithelium of seminiferous tubules contain only one or 2 layers of germinal cells. Polynucleated large round cells in advanced stages of dissolution may occur. Spermatids and sperm present. 2: Spermatogonia, spermatocytes and some spermatids present. Many spermatids are in stages of degeneration, and polynucleated cells may be formed by fusion of degenerating spermatids. Mature sperm are absent, whereas scattered maturing sperm may be seen. 3: Spermatogonia, spermatocytes, spermatids and a few sperm present. 4: Epithelium of seminiferous tubules appears normal, but number of sperm in lumen of tubules is less than normal. 5: Complete recovery.

‡ Calculated as elaidic acid.

unsaturation. $k_{233} = 0.65$ and $k_{268} = 0.04$. Methyl linolenate (*cis*-9, *cis*-12, *cis*-15 octadecatrienoic acid methyl ester) was prepared by Dr. L. C. Norcia. Its iodine value was 251.5, $k_{233} = 1.6$ and $k_{268} = 0.09$. The infrared spectrum detected the presence of a small amount of *trans* unsaturation. Linseed oil had low ultraviolet absorption ($k_{233} = 0.4$ and $k_{268} = 0.08$) and its infrared spectrum showed no evidence of *trans* unsaturation.

Results. Growth. The fat-free diet allowed a weight gain during the period of supplementation only about half that caused by linseed oil or linolenate supplementation, and about two-thirds that caused by linoleate. Compared with rats fed fat-free diet, those fed linolelaidate or *cis, trans* linoleate grew very slowly. This growth inhibition was overcome in both cases by supplements of linseed oil. Elaidate allowed growth better than that allowed by the fat-free diet. Thus the single *trans* double bond in a monoenoic acid does not inhibit growth, whereas in the nonconjugated diene, linoleate, one or more *trans* double bonds induces inhibition. Previous studies of the action of these compounds with much older rats did not demonstrate actual inhibition of growth, but these acids were found unable to stimulate growth in the older deficient rats(3,4). In the present study, the effect is more striking.

Skin condition. The skin symptoms were worsened by supplementation with linolelaidate or *cis, trans* linoleate. Previous experiments with older rats had demonstrated the inability of these unnatural isomers to cure skin symptoms. The worsening of the skin symptoms was prevented by linseed oil when it was given with the unnatural isomers.

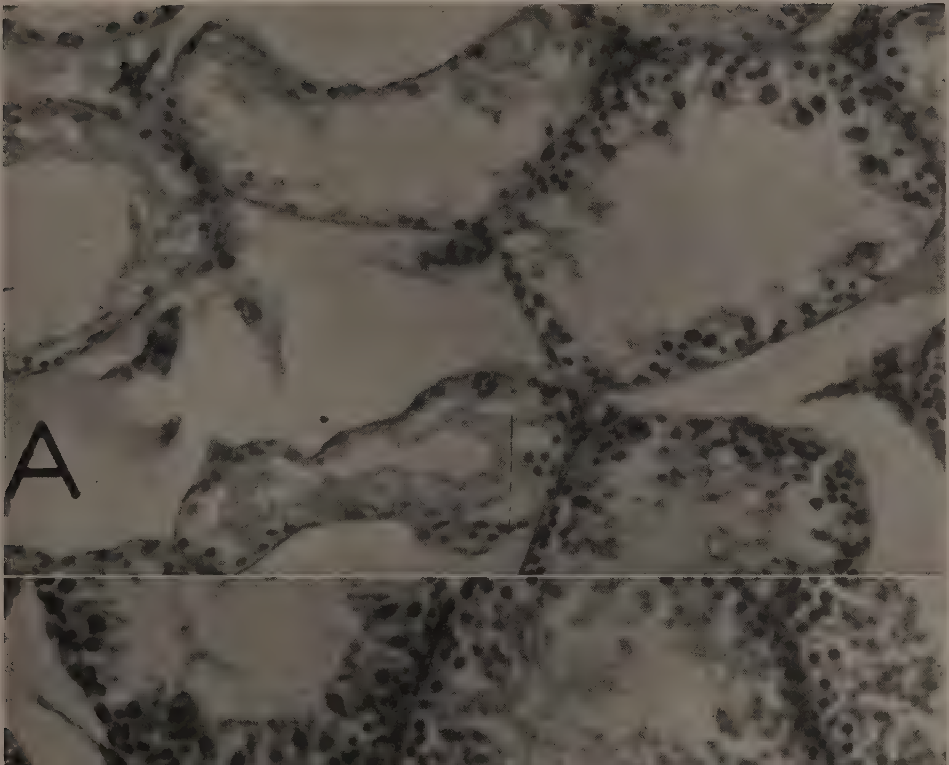

FIG. 1. A. Degeneration of spermatogenic tissue as result of EFA deficiency accelerated by cholesterol. Section of testis from rat No. 18. B. Recovery of spermatogenic tissue as consequence of supplementation with linseed oil. Section of testis from rat No. 16. Hematoxylin-Eosin stain. Magnification 67×.

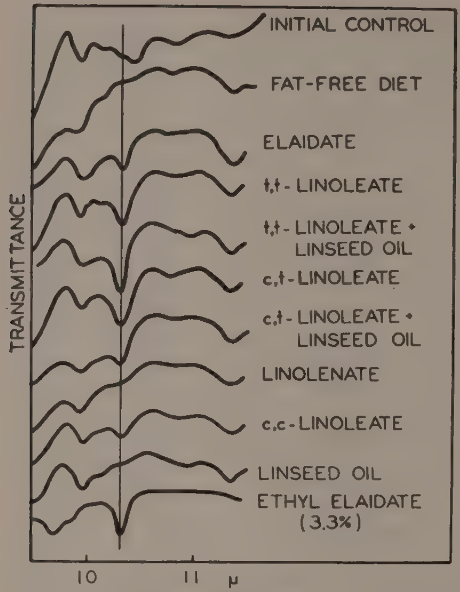

FIG. 2. Infrared spectra of fatty acid ethyl esters from rats fed various supplements. Spectra measured on 50% solutions of ester in carbon disulfide and compared with 3.3% ethyl elaidate + 46.7% ethyl oleate in carbon disulfide (lower curve).

Improvement of skin condition was significant only in the rats fed linoleate.

Testis weight. Testis weights of rats fed the *trans* isomers of linoleate were low. The ratio of testis weight to body weight was somewhat low in rats fed linolelaidate, in one rat fed *cis, trans* linoleate, in one fed *cis, trans* linoleate plus linseed oil and in one fed the fat-free diet. In all other cases only minor variations were found.

Histology of testis. That cholesterol increases the depletion of essential fatty acids is also indicated by the severe degeneration of spermatogenic tissue in the depleted rats. In a previous experiment weanling rats fed a fat-free diet 18 weeks had almost normal testicular tissue upon histological examination, but in the epididymi considerable numbers of degenerate spermatogenic cells were present (1). In the present study spermatozoa were not found in the epididymi of any of the rats. The epididymi were empty or contained only a few degenerate spermatogenic cells. Degenerated testes and the effect of feeding linseed oil are shown in Fig. 1. Supplementation of the fat-free diet with either elaidate or linolelaidate did not result in recovery of the spermatogenic tissue (Table I). Best recoveries were found in rats supplemented with linseed oil, and in one rat fed linoleate. Linolenate had no effect upon the spermatogenic tissue. The effect of *cis, trans* linoleate upon recovery of spermatogenic tissue is questionable.

Deposition of trans acids. The infrared spectra of methyl esters of the fatty acids of one rat from each group showed differences (Fig. 2). At the end of the depletion period there was *trans* unsaturation in the fatty acids of the rat. The sample taken from the fat-free rat at the end of the period of supplementation showed no evidence of *trans* double bonds, nor did the sample from the rat fed linseed oil. Fatty acids from the rats fed linoleate or linolenate had 1.2 and 0.9% of *trans* acids respectively, but the fatty acids from rats fed the unnatural *trans* isomers contained two or three times this amount. When the weight gain is related to the amount of *trans* unsaturation (expressed as elaidic acid), it is clear that the *trans* monoenoic acid does not have the growth inhibiting effect of *cis, trans* or *trans, trans* dienoic acids. It also appears that all the *trans* acids inhibited fatty acid synthesis.

Summary. Feeding weanling male rats a diet free of essential fatty acid (EFA), containing 1% cholesterol, 2% sulfasuxidine, and 1% hydrogenated coconut oil for 3.5 months induced an EFA deficiency accompanied by severe testicular degeneration. The degeneration was partially corrected by supplementation with linoleate or linseed oil for 3 weeks. However, sperm were not found in the epididymis in any case. *Trans* isomers of linoleic acid were unable to induce recovery of the testis. The *trans* isomers inhibited growth, worsened the skin condition, and were found to be deposited in the animal fat.

We are grateful to Dr. Jacques Chipault for aid in interpreting the infrared spectra and to Dr. James Peifer and Dr. J. P. Funch for valuable consultations.

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1. Aaes-Jørgensen, E., Funch, J. P., Engel, P. F., and Dam, H., *Brit. J. Nutrition,* in press.

2. Sreenivasan, B., and Brown, J. B., *J. Am. Oil Chem. Soc.*, 1956, v33, 341.

3. Holman, R. T., Proc. Soc. Exp. Biol. and Med., 1951, v76, 100.

4. Privett, O. S., Pusch, F. J., and Holman, R. T., *Arch. Biochem. Biophys.*, 1955, v57, 156.

5. Holman, R. T., *Arch. Biochem.*, 1947, v15, 403.

6. Peifer, J. J., and Holman, R. T., *Arch. Biochem. Biophys.*, 1955, v57, 520.

7. Kass, J. P., and Burr, G. O., *J. Am. Chem. Soc.*, 1939, v61, 1062.

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Histopathological Changes by Acetoacetate in Normal and Scorbutic Guinea Pigs.* (22700)

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Acetoacetate, which on repeated injection has been reported from this Laboratory to induce experimental diabetes by causing inactivation of insulin(1), hyperglycemia accompanied with reduced glucose tolerance(2), depletion of blood GSH(3), depletion of thiamin, riboflavin and niacin(4,5), has recently been found to cause hypovitaminosis C(6), which is also known to bear a close relation with diabetes(7,8). It has also been shown that disturbance in carbohydrate metabolism brought about in scurvy(9) is further aggravated by continued injection of acetoacetate in gradually increasing doses(10). Now because acetoacetate causes depletion of blood ascorbic acid when injected in high doses and histopathology in scurvy has been shown by Mukherjee and Banerjee(11) to reveal widespread degenerative changes in liver, spleen, kidney, testes and suprarenal cortex of chronic and acute scorbutic guinea pigs and since β-hydroxy butyric acid, another member of the ketone bodies has been shown by Nath *et al.*(12) to bring about histological changes in the pancreas, it seemed desirable to study the histopathological changes of different tissues of normal as well as scorbutic guinea pigs injected with acetoacetate in gradually increasing doses.

Methods. Fifty male guinea pigs weighing from 300 g to 400 g were divided into 4 groups, each group containing animals of approximately corresponding weights. The last group of animals served as the control, each animal being fed 5 mg ascorbic acid daily supplementing the recommended scorbutic diet (Group I—Control). Group II animals received diet like that of Group I and plus daily injections of acetoacetate (Sodium salt solution of concentration 200 mg/cc at pH 7.2) intraperitoneally, in increasing amounts. The initial dose was 175 mg/kg, increased by 40 mg/kg every third day so that final dose after 21 days was 415 mg/kg (Group II—Acetoacetate injected). Scurvy was produced in animals of Group III by feeding scorbutic diet as recommended by Banerjee(13). Animals of Group IV were fed the scorbutic diet and injected daily with acetoacetate in dosage similar to that in Group II (Group IV—Scorbutic and superimposed with acetoacetate injection). Animals were housed in individual cages. Paired feeding technic was followed to obviate any results due to inanition. The animals of Group II served as the control for this purpose, as it was seen from a preliminary experiment that the diet intake of this group was found to be the lowest as the experiment advanced. The animals of this group were fed the scorbutic diet *ad libitum* and the amount consumed by each was noted every day. The same amount of diet was fed, to every animal of corresponding weight in all the other groups, on the subsequent day. Just after decapitation different tissues of the animals of each group were removed and fixed in Zenker-formol solution. After dehydration

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and clearing, tissues were embedded in paraffin and 4-6 μ thick sections were cut and stained by haematoxylin and eosin stain. Pancreas were stained by Gomori's Phloxin-Haematoxylin method(14). Photomicrographs of different sections were taken under identical conditions.

Results. Histopathological changes in different groups of animals have been presented separately. Pancreas of the Group II animals (acetoacetate injected for 21 days) show an increase in the area of the islet tissues and the number of β-cells. Group III animals (Scurvy) show distinct early hypertrophy and early degranulative changes of the pancreatic islet cells but no degenerative changes. Group IV (Scurvy superimposed by repeated injection of acetoacetate) show more clear changes. Fibrosis and degranulation are more distinct. Hypertrophy of the islet cells and pyknotic changes in the nuclei, which precede degenerative symptoms of the β-cells are also evident. Animals of this group also show clear evidence of small degree of lipomatosis.

Kidney of Group II animals (acetoacetate injected) show hypertrophy of the glomeruli and early hyalinization of the proximal convoluted tubules. Changes in the tubular cells and nuclei are indicative of hyperfunction. Group III animals (scurvy) show atrophy of the glomeruli and degenerative changes in the tubules. Kidneys of the Group IV animals (scurvy superimposed by repeated injections of acetoacetate) show mild intercapillary glomerulosclerosis. Capsular cells are also distended. Proximal convoluted tubules show the presence of hyaline casts and necrosis.

Liver of the Group II animals (acetoacetate injected) show hydropic distension of the hepatic cells and pyknosis of a few nuclei around the central vein. In Group III animals (scurvy) hepatic cells show distinct signs of early degeneration. In some cases nuclei are found to be pushed to the periphery of the cells. Liver cells of the Group IV animals (scurvy superimposed by repeated injections of acetoacetate) show characteristic necrotic changes in the peripheral zone of the lobule. Hemorrhage of the hepatic cells is also seen around the central vein.

Discussion. Previous studies have shown that repeated injections of acetoacetate to guinea pigs kept on scorbutic diet cause a rapid development of hyperglycemia and depletion of glycogen storage of liver and leg muscle(10). Continued injection of acetoacetate alone can also cause different changes in carbohydrate metabolism similar to that observed in diabetic condition. Scurvy has been known to cause degranulation of the β-cells of the pancreas(15), and to lead to various disturbances in carbohydrate metabolism(9). Repeated injection of acetoacetate has also been found to destroy vit. C *in vivo* (6). The role of acetoacetate in the aggravation of the disturbances of carbohydrate metabolism in scorbutic condition(10) has thus been accounted for.

The present study of the histopathological changes in different tissues of the animals kept on scorbutic diet and simultaneously injected with repeated doses of acetoacetate is in keeping with the biochemical lesions reported earlier(10). Pancreas of the scorbutic animals show early degranulative changes. Acetoacetate injection alone can also cause hypertrophic changes but when both conditions are superimposed, histopathology of the pancreatic islet cells is considerably aggravated showing fatty infiltration and early fibrosis of the tissue and pyknotic changes of the nuclei indicating overactivity and exhaustion. Kidney changes also present similar results. Acetoacetate injection alone causes hypertrophy, showing hyperfunction of the tissue, indicating more work for the cortex to metabolize acetoacetate. Degeneration and atrophy of the tubules begin with scurvy; but scurvy superimposed with the injection of acetoacetate lead to intercapillary glomerulosclerosis a condition which appears commonly in clinical diabetes(16).

Hydropic degeneration of liver cells in acetoacetate treated animals may be due to toxicity of acetoacetate injections. Scorbutic hepatic cells show early degenerative changes, which are aggravated leading to necrosis and haemorrhage around the central vein when the acetoacetate injections are superimposed on scorbutic condition.

All these findings demonstrate the toxicity of acetoacetate injections upon the structural integrity of the tissues. They also confirm and explain the biochemical findings as regards the aggravation of disturbances in scurvy by acetoacetate(10). The accumulative disturbances in carbohydrate metabolism thus produced finally tend to affect the architecture of the pancreatic islets accompanied with the degeneration of the nuclei of the β-cells, commonly observed in clinical diabetes(17), thus confirming the deleterious effect of acetoacetate in inducing diabetes in experimental animals and disclosing a possibility of excess accumulation of acetoacetate serving as a prominent factor for gradual development of clinical diabetes.

Summary. The islets of Langerhans of scorbutic guinea pigs and those injected with acetoacetate were hypertrophied. Injection of acetoacetate into scorbutic animals caused further degranulation and degenerative changes. Acetoacetate brought about hypertrophy of renal cortex and scurvy caused atrophy of glomeruli. Combined treatment aggravated these degenerative changes. Both scurvy and acetoacetate caused central degeneration of liver and this is enhanced by the combined treatment. In agreement with histologic changes, biochemical findings indicate the toxicity of acetoacetate. Acetoacetate may be a prime factor in clinical development of diabetes.

1. Nath, M. C., and Brahmachari, H. D., *Nature* (*Lond.*), 1946, v157, 92.

2. Nath, M. C., and Chakrabarti, C. H., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 326.

3. Nath, M. C., Hatwaline, V. G., and Gadgil, J. S., *Biochem. J.*, 1953, v53, 479.

4. Nath, M. C., and Chakrabarti, C. H., *Ind. J. Med. Res.*, 1953, v41, 257.

5. ———, PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 5.

6. ———, *ibid.*, 1951, v78, 369.

7. Pfleger, K., and Scholl, F., *Wien. Arch. inh. Med.*, 1937, v31, 219.

8. Ergele, L. K., *Arch. Kinderheilk*, 1939, v56, 101.

9. Banerjee, S., and Ghosh, N. C., *J. Biol. Chem.*, 1947, v168, 207.

10. Nath, M. C., Sahu, V. K., and Chitale, R. P., *Biochem. J.*, 1953, v53, 684.

11. Mukherjee, A. K., and Banerjee, S., *Anat. Rec.*, 1954, v120, 907.

12. Nath, M. C., Brahmachari, H. D., and Gopalkrishna, A., *Science*, 1950, v112, 92.

13. Banerjee, S., *J. Biol. Chem.*, 1945, v159, 327.

14. Gomori, G., *Am. J. Path.*, 1941, v17, 395.

15. Banerjee, S., *Nature* (*Lond.*) 1944, v153, 344.

16. Bell, E. T., *Diabetes*, 1953, v2, 376.

17. Best, C. H., *ibid.*, 1952, v1, 257.

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Purification of Pancreatic and Urinary Callicrein. (22701)

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The hypotensive substance callicrein (kallikrein or padutin) was first discovered by Abelous and Bardier(1) and systematically studied by Frey, Kraut Werle and co-workers for the next 3 decades(2-3). Procedures for the purification of both urinary and pancreatic callicrein(4-10), with the exception of that developed by Reisser(10), depend to some extent on the adsorption of impurities and/or callicrein on various colloidal salts. Since adsorption conditions are difficult to reproduce and frequently require the running of test samples before each step in the preparation,* the present method was devised based on an ethanolic fractionation scheme such as carried out by Cohn *et al.* upon plasma proteins(11) and utilized successfully by this laboratory for purification of hyaluronidases(12-13).

Methods. Callicrein content of the various fractions was determined by the method of

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* Werle, E., personal communication.

Frey *et al.*(2). Dogs were anesthetized with 26 mg nembutal per kilo and the blood pressure recorded by a mercury manometer attached to the carotid artery. Injections of test solutions were made through a polyethylene catheter inserted in the femoral vein. Heparinized saline (50 mg per liter) was used to prevent clotting in the catheter. Samples of pancreatic callicrein containing ¼ to 2 Frey units[†] gave a response which was complete within one minute and solutions could readily be assayed at the rate of one every 2 minutes particularly with more highly purified preparations. However, with crude urinary fractions the blood pressure frequently did not return to its original level making the quantitation of the results difficult. *Total nitrogen* was determined by micro-kjeldahl digestion and direct nesslerization. For determinations where ammonium sulfate was present, aliquots were made alkaline with NaOH and the ammonia removed by aeration prior to digestion. Samples similarly treated, but not digested, served as controls. *Electrophoretic examination* and separation were conducted at 0-1° by moving boundary methods in the Perkin-Elmer equipment and the Aminco-Stern apparatus. Samples were equilibrated by dialysis with 0.1 ionic strength phosphate buffer pH 7.7.

Results. Purification of pancreatic callicrein: Fresh frozen bovine pancreas was thawed by standing overnight at 4°, then minced by machine. Three methods of extraction (autolysis(9), 0.1 M acetic acid and 0.25 M trichloroacetic acid) showed that 0.1 M acetic acid extracted the greatest number of callicrein units although autolysis gave the most highly purified product. Initial crude extracts were therefore prepared by addition of an equal weight of chilled 0.1 M acetic acid to cold mince. The preparation was stirred for 1 hour at 4° and then centrifuged at 2000 rpm and 26°. The extract was further clarified by filtration on Buchner funnels through filter paper pulp. The filtrate was measured and 1/10 its volume of concentrated buffer added to make 0.02 M phosphate at pH 5.5 containing 0.45% NaCl and 1:10,000 merthiolate. From 5 kg of pancreas a final volume of around 4,500 ml was obtained, containing 2.5-3 Frey units and 30-40 mg protein per ml as calculated from nitrogen assay.

First ethanol fractionation: The crude extract was adjusted to pH 5.5 and fractionated with ethanol at —5°. The ethanol added was precooled at —20° and introduced in a thin stream with concurrent stirring of the cooled buffered solution. After addition of each increment of alcohol, the fraction was allowed to equilibrate for at least 18 hours before removal of precipitate by centrifugation at 4000 rpm and —5°. The precipitate was dissolved in the minimum amount of buffer at 0°. Preliminary fractionation using 0.05 *mole fraction* increments of ethanol demonstrated that 50% of the callicrein activity could be recovered in the precipitate between 0.15 and 0.25 mole fraction with a 6-fold increase in purity based on nitrogen determinations. However, occasional losses of callicrein, due to adsorption on the large precipitate obtained with 0.15 mole fraction, led to eventual adoption of a one step precipitation at 0.25 mole fraction. As illustrated in Table I, this one step procedure effected a 3-4 fold increase in purification and retained all callicrein activity of original extract.

Second ethanolic fractionation: Since callicrein was known to be stable from pH 5.0 to 8.0(14) a pH of 7.5 was chosen for the next ethanolic fractionation. Experimentation revealed that callicrein was now precipitated between 0.25 and 0.45 mole fractions. Precipitate obtained from the first fractionation was dissolved in 0.02 M phosphate buffer and diluted to 10-12 mg protein per ml. The pH was adjusted to 7.5 and precipitates removed following addition of 0.25, 0.3 and 0.45 mole fractions. Excessive adsorption of callicrein on relatively large precipitates obtained with 0.25 mole fraction was not noted at this concentration of protein (Table I). Precipitates obtained from 0.3 and 0.45 mole fraction were

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[†] The authors are deeply indebted to Dr. E. Werle, University of Munich, Germany, for supplying vials of standard callicrein containing 10 Frey units. These preparations contained 5.5-6 Frey units/mg and 45-55 Frey units/mg N.

TABLE I. Ethanolic Fractionation of Pancreatic Callicrein.*

Purification procedure	Conc. ethanol mole fraction	Total callicrein units	% recovered	Purity index, units per mg N
0.1 M acetic acid extract		12,800	100	0.5
1st ethanol fract. pH 5.5	.25	15,000	117	1.6
2nd ethanol fract. pH 7.5	.25	6,400	43	2.5
	.3	2,280	15	25
	.45	12,000	80	37
3rd ethanol fract. pH 6.5	.3	500	3.5	23.5
	.35	650	4.5	45
	.5	5,100	42.5	104
	.55	550	4.2	25

* Above data are based on avg figures obtained from five 5 kg batches of pancreas.

usually sufficiently small to warrant collecting in two centrifuge tubes before dissolving in buffer. This procedure was followed so that residual ethanol present in the cups would not destroy callicrein. The resulting solutions were about 20 times as pure as that obtained in first ethanol precipitation.

Third ethanolic fractionation: This fractionation was conducted on combined precipitates from 0.3 and 0.45 mole fractions from above. They were diluted in buffer to 0.5 to 0.75 mg protein per ml with the pH adjusted to 6.5. Even though the pH was nearer the isoelectric point (pH 4.0 to 4.3(2)), the decrease in concentration of protein being fractionated caused the bulk of activity to precipitate betwen 0.35 and 0.5 mole fractions (Table I). Following the three ethanolic fractionations approximately 5,000 Frey units could be obtained from 5 kg of bovine pancreas. The final preparation was approximately 200 times purer than initial crude extracts. Average yield of material calculated as protein was 400 mg. Callicrein of this purity was reasonably stable when stored at 4° and at 0.1% protein concentration. It is possible that variation in activity obtained from one preparation to another at this stage may in part reflect a certain instability. Crude acetic acid extracts could be dialyzed against running tap water without inactivation. However, material from the third ethanolic fractionation lost 50% of its activity in 24 hours dialysis. It could be dialyzed against buffer at 4° without loss.

Electrophoretic examination and separation. In order to obtain sufficient protein concentration for electrophoretic examination, 6,500 callicrein units which had been obtained by 3 ethanolic fractionations and which gave an average purity of 75 units per mg N were recombined in 350 ml, pH was adjusted to 5.5, and 0.3 mole fraction of ethanol added. The precipitate was extracted with 3 successive 12 ml portions of 0.1 ionic strength phosphate buffer pH 7.7. Approximately 75% of the enzyme was recovered in 3 solutions with 2-fold increase in purity for the first extract. The 3 solutions were separately dialyzed against buffer at 4°. No loss in total enzymatic activity was noted. The first extract showed another 2-fold increase in purity, the second extract went from 35 units per mg N to 150 units per mg N, while the third extract remained unchanged at 60 units per mg N. Each of the 3 solutions were analyzed electrophoretically by moving

TABLE II. Electrophoretic Separation of Partially Purified Callicrein.

	Total callicrein units	Purity index, units/mg N	Relative % of electrophoretic components					
			1	2	3	4	5	6
Extract No. 1	100	300	47	18	11	24	—	—
Ascending	75	430	69	19	6	6	—	—
Descending	7.5	85	18	18	18	45	—	—
Extract No. 2	850	150	27	22	6	13	8	23
Ascending	540, 540*	330, 297	42	29	6	11	4	8
Descending	61, 46	35, 37	14	16	6	15	11	38
Extract No. 3	100	60	14	9	2	5	5	65
Ascending	35, 75	320, 400	42	23	4	9	5	17
Descending	15, 30	38, 52	5	4	1	4	5	81

* Values represent duplicate determinations on same extract.

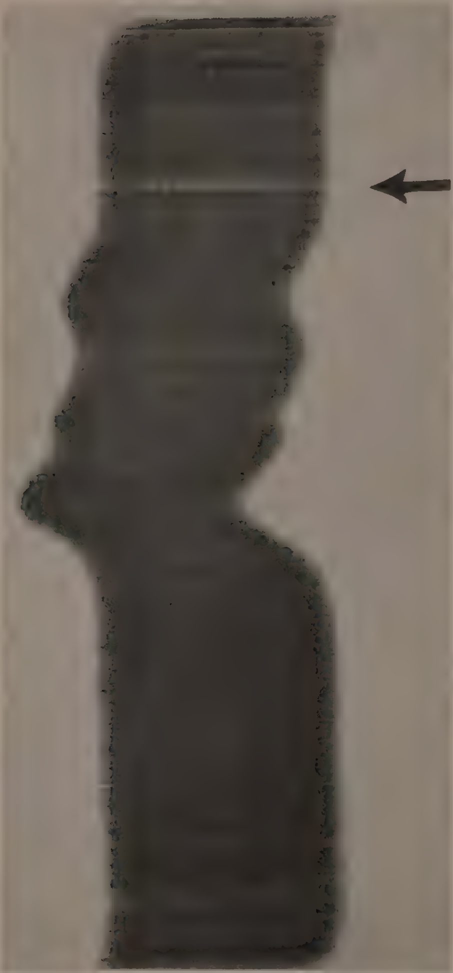

FIG. 1. Ascending electrophoretic pattern of partially purified callicrein (Extract No. 1). Time: 1920 sec. Volts: 5.0 volts/cm. Arrow indicates starting point.

boundary technic (Fig. 1) and then separated into 2 fractions, one containing the major portion of the fast component and the other the slow material with the intermediate substance being divided between the 2 fractions. As is shown in Table II, the sample with highest purity index, extract 1, showed 4 components with mobilities ranging from about —6 to —3 x 10^{-5}cm^{2}sec^{-1}volt^{-1}. The fastest component (as estimated from distribution pattern areas) accounted for 47% of the original material and the slowest 24%. Electrophoretic separation increased the concentration of fast material to 69%. The other two extracts contained 6 or more components varying in mobility from —6 to —0.5 x 10^{-5} cm^{2}sec^{-1}volt^{-1}. Their decrease in purity index paralleled the decrease in content of fast material and the corresponding increase in slower moving components. In addition, the activity of preparations following separation was invariably associated with the fast component. Callicrein, therefore, is tentatively identified as the most rapidly migrating substance present in these solutions. Activity of these purer preparations of callicrein, rapidly lost at room temperature, could be maintained only by storage of samples in frozen state. Further purification by moving-boundary procedure did not appear feasible since mobilities of various components were similar and diffusion rates were fast. Paper electrophoresis at 4° and 25°, could also be used for further purification of this enzyme. However, considerable loss was incurred both from irreversible adsorption on paper and by inactivation during time period required for the experiment.

Ammonium sulfate fractionation. Ammonium sulfate fractionations were carried out at 4° and individual precipitates were allowed to equilibrate for 24 hours before being removed by centrifugation at 4000 rpm, 4°. As is shown in Table III levels of purity were obtained which compare favorably with those achieved by electrophoretic separation. However, callicrein of this purity again readily lost activity at room temperature. The technic of adding albumin to maintain high protein concentrations which had been successfully used in stabilization of hyaluronidase up to 100,000 T.R.U. per mg N(15), failed

TABLE III. Ammonium Sulfate Fractionation of Partially Purified Pancreatic Callicrein.

Saturation of $(NH_4)_2SO_4$	Total callicrein units	% recovered	Purity index, units/mg N
.3	750	7.5	10
.4	1,200	12.0	50
.5	4,950	49.5	450
.6	1,500	15.0	300
Supernatant	750	7.5	5

to prevent inactivation of the hypotensive enzyme.

Purification of urinary callicrein. Methods originally devised for purification of bovine pancreatic callicrein were subsequently applied to purification of urinary callicrein from human urine. Urine was collected and stored for short periods of time under xylene. Addition of suitable amounts of 0.02 M phosphate buffer was made and pH adjusted to 5.5. Preliminary fractionation indicated that despite the increased dilution of enzyme in solution (0.075 Frey unit per ml as compared to 2.5-3.0 per ml for crude pancreatic extract) 90-100% of the enzyme was precipitated by the 0.25 mole fraction of ethanol. However to insure complete precipitation of callicrein 0.3 mole fraction was utilized. In 100 liters of human urine used in present studies, the average number of callicrein units was 0.75 Frey unit per 10 ml. This number of units is within the range found by previous investigators(2).

Callicrein in 40 liters of normal urine was concentrated by precipitation at pH 5.5 with 0.3 mole fraction. The solution was then diluted to 5 mg protein per ml as calculated from nitrogen assay, the pH adjusted to 7.5 and ethanol added to 0.15 mole fraction. Although the bulk of activity failed to precipitate until 0.3 mole fraction of ethanol, this stepwise separation was utilized in order to minimize loss by adsorption. The ethanol concentration was then raised to 0.25 mole fraction and a second inactive precipitate was removed. Precipitates from 0.3, 0.35 and 0.4 mole fractions were also obtained. As is shown in Table IV, the 0.35 fraction contained half the starting activity with better than a 50-fold increase in purity. Urinary callicrein differed from pancreatic in that precipitation at pH 7.5 occurred at a lower mole fraction despite an increased dilution of enzyme in solution (0.7 Frey unit per ml as compared to 3 units for pancreatic preparation).

Discussion. Although originally reported to be present in relatively large amounts in the saliva, pancreas and urine, more recent evidence by Werle and Maier(3) suggests that pancreatic and urinary preparations are chemically and pharmacologically different from the submaxillary substance. Callicrein is reported to exert its hypotensive effect by enzymatic action on callodinogen, present in normal plasma, to release a smaller molecule, presumably polypeptide, called callidin. In this manner the callicrein-callidin system with respect to hypotension can be compared to the renin-hypertensin (angiotonin) mechanism which has been associated with hypertension(16).

TABLE IV. Purification of Urinary Callicrein.

Purification procedure	Conc. ethanol mole fraction	Total callicrein units	% recovered	Purity index, units per mg N
Normal human urine		3,000		.01
1st ethanol fract. pH 5.5	.3	2,880	96	1.0
2nd ethanol fract. pH 7.5	.15	47.3	1.6	1.0
	.25	100	3.5	1.6
	.3	280	9.7	14
	.35	1,440	50.0	57
	.4	160	5.5	10

Callicrein has previously been obtained at high levels of purity by two authors(5,7) who utilized a variety of technics. The purity achieved by Bischoff and Elliot(5) from human urine is probably similar to that obtained by us with pancreatic callicrein. However, Kraut and Schweitzer(7) have reported preparation of callicrein solutions containing 2 micrograms of organic material per callicrein unit. Calculation of these data based on the assumption that a pure protein had been isolated would give a purity index of 3000 Frey units per mg N. On the other hand, if the fast component in electrophoretic analysis at pH 7.7 can be taken as a measure of callicrein, then a solution containing 700 Frey units per mg N should represent a homogenous preparation. The discrepancy between these calculations is so great that further work would be necessary to elucidate the difference. Those authors using bovine pancreas(9-10) attempted only preliminary purification of the callicrein. While it is undoubtedly true that more units of callicrein can be obtained by autolysis than by extraction with dilute acetic acid, in our hands, as well as those of Reisser(10), autolysis at 37°

gave secondary reactions which were undesirable and caused inconsistent results. The autolysis devised by Reisser which takes place for 72 hours at 2° has not been attempted in this laboratory since it was desired to take all possible precautions against contamination of the extracts. However, this method of extraction should cause a 4-fold increase in the recoverable units.

The isolation of callicrein as a chemical entity would appear to depend on the finding of a method for stabilizing this hypotensive substance. The instability encountered in our preparation at around 400 Frey units per mg N and those reported by other authors(5,7) has hampered further attempts to characterize this enzyme.

Summary. A method has been devised for preparation of highly purified pancreatic callicrein. The fractionation procedure involves three ethanolic fractionations at pH 5.5, 7.5, and 6.5 followed by one ammonium sulfate precipitation. Solutions of hypotensive enzyme at this level of purity proved highly unstable and further purification was abandoned. Preliminary purification of urinary callicrein revealed that the method could be adapted to this source.

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1. Abelous, J. E., and Bardier, E., *C. R. Soc. Biol.*, 1908, v64, 848.

2. Frey, E. K., Kraut, H., and Werle, E., *Kallikrein, Padutin, Stuttgart,* 1950.

3. Werle, E., and Maier, L., *Biochem. Z.*, 1952, v323, 279.

4. Kraut, H., Frey, E. K., Bauer, E., and Schultz, F., *Z. Physiol. Chem.*, 1932, v205, 99.

5. Bischoff, F., and Elliot, A. H., *J. Biol. Chem.*, 1935, v109, 419.

6. Werle, E., and Marcus, A., *Biochem. Z.*, 1938, v296, 275.

7. Kraut, H., and Schweitzer, I., *Z. Physiol. Chem.*, 1949, v284, 211.

8. Frey, E. K., and Werle, E., *ibid.*, 1930, v189, 97.

9. Farbenindustrie, I. G., (Fr. patent No. 843,423), 1939, July 3.

10. Reisser, O., *Biochemica and Biophysica Acta*, 1947, v1, 147.

11. Cohn, E. J., Stung, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, v68, 459.

12. Freeman, M. E., Anderson, P., Webster, M. E., and Dorfman, A., *J. Biol. Chem.*, 1950, v186, 201.

13. Baker, E. M., Webster, M. E., Freeman, M. E., Cary, S. C., and Sanders, A. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 24.

14. Bischoff, F., and Elliot, A. H., *J. Biol. Chem.*, 1933, v100, XVII.

15. Freeman, M. E., and Webster, M. E., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 113.

16. Best, C. H., and Taylor, N. B., *The Physiol. Basis of Med. Prac.*, Baltimore, 1950.

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Tolerance of the Rat for N,N′-Diphenyl-P-Phenylenediamine. (22702)

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The demonstration that N, N′-diphenyl-p-phenylenediamine (dppd) is effective in the prevention of encephalomalacia in chicks fed vit. E-low diets containing fish oils, and in the stabilization of carotene and vit. A in practical rations, has led to its widespread use in commercial mixed feeds. The concentration commonly employed is 0.025% of the diet. Matterson *et al.*(6) have reported that chicks which received a diet containing 1% dppd throughout a 12-week growth trial remained free of serious lesions and maintained a normal growth rate. Draper and Johnson (1) observed no adverse effects from administering dppd to lambs at a level of 0.1% for 7 weeks, and Jensen and coworkers* reported that 0.025% dppd improved reproductive performance in turkey hens fed a tocopherol-low ration.

The present experiments were designed originally to determine the degree of replace-

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* Abstract published.

TABLE I. Reproductive Performance of Rats Receiving a Vit. E-Free Diet Supplemented with dppd from Weaning Age.

Treatment	No. females	No. conceptions	No. pups born — Total	No. pups born — Per conception	% born alive
0	25	0	0	0	—
Vit. E (30 mg/wk)	19	17	155	9.1	57
.1% dppd	10	7	21	3.0	19
.025% "	14	10	71	7.1	7
.005% "	16	16	108	6.8	14
.385 mg " /wk*	10	9	69	7.7	88

* Approximately .0006% of diet.

ability of vit. E by dppd in the rat; but following the observation that small concentrations of dppd fed continuously through growth and gestation produced adverse effects upon reproduction, a study of the long-term consequences of dppd feeding was conducted.

Methods. Weanling female rats of the Sprague-Dawley strain were maintained throughout growth and reproduction on a diet of the following composition (%): Cerelose 64.6, casein 20, tocopherol-low lard 10, salts(5) 4, choline 0.4, vitamin premix (in cerelose) 1. Vit. A and D were administered weekly by dropper. The premix provided the following concentrations of vitamins in μg per gram of diet: Thiamin 100, riboflavin 15, nicotinic acid 100, pyridoxine 6, calcium pantothenate 40, biotin 0.5, menadione 4, folic acid 4 and vit. B_{12} 0.05. Comparable groups of rats were fed the basal diet alone or supplemented with alpha-tocopheryl acetate (approximately 30 mg/wk placed on the diet), or dppd (0.1%, 0.025%, or 0.005%). Other animals received .385 mg dppd per week in 3 equal oral doses (equivalent to approximately 0.0006% of the diet eaten).

Results. During the growing period of 6 weeks all animals gained normally and remained in healthy condition with no manifestations of dppd intoxication or vit. E deficiency. The females, weighing 175-200 g, subsequently were mated to healthy males which had been maintained on a stock diet.

During gestation the females receiving 0.1% dppd exhibited anorexia, rough haircoat and anemia. At parturition gross hemorrhages from the vagina were observed and, upon autopsy, hemorrhages were located in the uterus. Of 10 females, 3 succumbed to hemorrhages and the remainder were in semimoribund condition. The hemorrhagic animals were acutely anemic but the prothrombin times were normal. Seventeen dead and 4 live pups were obtained from this group.

Improvement in reproductive performance was observed with lower concentrations of dppd in the diet. These results are summarized in Table I. Litter size was comparable among the groups receiving the 3 lowest levels, but the proportion of live pups was subnormal except at the lowest concentration. No abnormalities were found in the hemoglobin values, prothrombin times or white blood cell counts during gestation. However, the feeding of dppd appeared to be associated with an increase in the gestation period, the average for the females receiving 0.025% dppd being 25.4 days and that for the 0.005% level being 23.8 days, compared with 22.8 days for the positive controls.

The efficacy of dppd in reversing sterility in females maintained on a vit. E-low diet was also examined. The females were depleted of vit. E using a diet similar to that outlined but containing 4% fish liver oil as a source of fat. Methyl linoleate was given orally twice per week at a rate equivalent to 0.4% of the diet. All the females exhibited reproductive failure during the first cycle. Supplementation was begun one week before remating. The results are shown in Table II.

It has been reported previously that dppd fails to regenerate vit. E-deficient female rats fed a diet essentially devoid of this vitamin, although, at a level of .385 mg per week given prophylactically, dppd can delay the appearance of resorption-gestation for one or two cycles(2,3). The regenerative effect observed

TABLE II. Efficacy of dppd in Restoring Fertility of Female Rats Previously Maintained on Vit. E-Low Diet.

Treatment	No. females	No. conceptions	No. pups born — Total	No. pups born — Per conception	% born alive
0	22	0	0	0	—
.1% dppd	19	15	71	4.7	3
.01% "	17	17	109	6.4	5
Vit. E	23	19	161	8.5	71

in terms of pups born in this experiment is probably attributable to its protective action on the small quantity of vit. E contributed by the fish liver oil. The oil, upon assay by the procedure of Tosic and Moore(4) was found to contain 8 mg/100 g, and contributed, therefore, about 3.2 μg/g of diet. These data indicate that the dietary requirement of the rat for vit. E may be considerably reduced when an effective antioxidant is supplied simultaneously, and support the concept of a dual role for vit. E in the body, including a general antioxidant function and another indispensable role in whch it cannot be replaced by other antioxidants. The inferior survival *in utero* on both concentrations of dppd in this regeneration experiment further substantiates the long-term toxic effect of this compound at levels well below those used in commercial feeds. However, only slight indications of hemorrhaging at parturition were observed in the animals receiving 0.1% dppd, possibly because of the shorter period of administration. The possibility that levels below .01% might exert a regenerative effect is being investigated.

Weanling rats showed no adverse effects from receiving 0.5% dppd in the diet for 12 weeks, but subsequently developed "porphyrin" staining of the whiskers and haircoat over the head and neck. This condition was accompanied by a rough haircoat and moderate depression of weight gain. Determinations of hemoglobin values and prothrombin times on blood from these animals have revealed no significant changes. Food consumption and growth rate during the early part of the growing period indicate that palatability was not a problem.

In the majority of these experiments the dppd employed was a feed grade product containing approximately 95% active material. However, part of the growth and reproduction studies were conducted using pure dppd, and indicate that the toxicity also is encountered after prolonged feeding of the pure compound and is not attributable to a contaminant in the feed grade product.

The results of this study show that prolonged continuous oral administration of dppd in a vit. E-low diet at levels substantially below those used commercially in mixed feeds is toxic for the rat. The symptoms do not appear during growth, except at very high concentrations, but occur at parturition, and are characterized mainly by vaginal hemorrhages in the female and a high incidence of stillbirths. The possibility that this toxicity may be significantly modified by the presence of vit. E in the diet is worthy of investigation but may be regarded as unlikely.

Summary. Inclusion of N, N′-diphenyl-p-phenylenediamine in a vit. E-low diet fed to female rats through growth and reproduction has resulted in varying degrees of reproductive failure. Toxicity was encountered during growth at a level of 0.5% but not at 0.1% or below. Rats receiving 0.1% dppd exhibited uterine and vaginal hemorrhages at parturition accompanied by anorexia, anemia and emaciation. Concentrations of 0.025%, 0.01% and 0.005% resulted in a high incidence of stillbirths. No adverse effects were encountered from feeding 0.0006% dppd in the diet. The data show that dppd elicits symptoms of toxicity in the rat at concentrations below those used in commercial mixed feeds.

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1. Draper, H. H., and Johnson, B. Connor, *J. Animal Sci.*, 1955, v14, 1200.

2. Johnson, B. Connor, and Goodyear, S., *Terzo Congresso Internazionale della Vitamina E, Venice,* 1955, I. Prodotti Roche S.P.A., Milan, Italy, p46-47.

3. Johnson, B. Connor, *Vitamina E. Atti del Congresso Internazionale Venezia,* 1955. Edizioni Valdonega Verona, p456-57.

4. Tosic, J., and Moore, T., *Biochem. J.,* 1945, v39, 498.

5. Spector, H., *J. Biol. Chem.,* 1948, v173, 659.

6. Matterson, L. D., Potter, L. M., Kozeff, A., and Jungherr, E. L., *Poult. Sci.,* 1955, v34, 239.

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Metabolic Properties of Quinidine; Effects of Quinidine Sulfate on Anaerobic Carbohydrate Metabolism of Rat Diaphragm.[*] (22703)

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Several papers have appeared recently concerning the effect of quinidine on various aspects of carbohydrate metabolism(1,2,3). The influence of this compound on the anaerobic phase of carbohydrate metabolism has not been thoroughly explored, and results which appear to be conflicting have been reported. Following the work of Furman and Howard(4) confirmed by Fajans and his group(5) who showed that quinidine decreases glucose tolerance in human subjects, we decided to investigate the influence of this drug on the anaerobic metabolism of rat diaphragm in an attempt to gather information on the mechanism of this effect.

The combined results of these experiments on the effect of quinidine on endogenous glycolysis, on glycolysis in the presence of glucose and fructose and on anaerobic consumption of glucose and fructose, point to the conclusion that utilization of these hexoses is impaired at one or more steps close to their entry into the Embden-Meyerhof sequence.

Materials and methods. Albino rats (Holtzman) weighing from 90 to 130 g were used. Following a 24-hour fast the rats were killed by a blow on the head and the diaphragms quickly removed and placed in Krebs-Ringer's bicarbonate solution at 1°C. The diaphragms were cut into either 2 or 4 pieces, weighed and used as soon as possible. All experiments were carried out under anaerobic conditions using the standard Warburg procedure. Incubation was carried out at 37°C in Krebs-Ringer's bicarbonate solution with or without quinidine sulfate (U.S.P., Merck). The flasks were gassed one-half hour with 95% nitrogen-5% carbon dioxide. After an equilibration period of 15 minutes glucose (Baker) or fructose (Nutritional Biochemicals) dissolved in Krebs-Ringer's bicarbonate solution was added from the side bulb to give a final total volume of 3.0 ml. Glucose or fructose was determined by the Somogyi-Nelson method (6).

Results. Effect of quinidine on anaerobic uptake of added glucose and fructose. Glucose (4 mg/ml) and fructose (5 mg/ml) uptake was determined, each in a series of 5 experiments, with or without 1 x 10^{-3} M quinidine. The following mean values and standard errors were obtained: glucose consumption without quinidine, 2.74 ± 0.21 mg/g (wet wt) tissue/hr; with quinidine, 1.44 ± 0.16 mg/g/hr; fructose consumption without quinidine, 2.70 ± 0.27 mg/g/hr; with quinidine, 1.28 ± 0.08 mg/g/hr. The inhibition of glucose or fructose uptake produced by 1 x 10^{-3} M quinidine is significant at the 0.01 level. In 2 experiments in which glucose consumption was measured for a 30-minute pe-

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TABLE I. Effect of Quinidine Sulfate on Anaerobic Glycolysis of Rat Diaphragm. Figures are mean values and ranges for CO_2 production in μl/g tissue (wet wt)/hr in Krebs-Ringer's bicarbonate solution. Incubation period 2 hr.

Substrate, mg/ml	No. of exp.	No quinidine		Quinidine concentration	Quinidine		Change in endogenous	Stimulation by substrate	Stimulation by substrate
		Endogenous	With substrate		Endogenous	Substrate	With quinidine (%)	No quinidine (%)	Quinidine (%)
Fructose 5	8	100 (77–133)	109 (84–135)	1×10^{-3} M	94 (70–123)	93* (67–106)	-6	+ 9	− 1
Glucose 1	2	112 (106–118)	162 (155–169)	5×10^{-4} M	105 (99–110)	130 (125–135)	−6	+ 45	+24
" 1	3	90 (80–99)	132 (108–178)	1×10^{-3} M	88 (83–94)	83 (71–92)	−3	+ 47	− 6
" 4	2	70 (63–77)	144 (122–167)	1×10^{-3} M	73 (68–77)	78 (75–81)	+4	+106	+ 7

* 5 experiments.

riod the inhibition was less marked: 7.10 mg/g/hr without quinidine; 4.80 mg/g/hr with quinidine.

Effect of quinidine on lactic acid production. The effect of quinidine on the endogenous anaerobic $Q_{CO_2}^{N_2}$ of rat diaphragm and on $Q_{CO_2}^{N_2}$ in the presence of added glucose and fructose is shown in Table I. Endogenous lactic acid production is not affected by 1 x 10^{-3} M quinidine. The results indicate a marked stimulation of lactic acid production by added glucose at concentrations of 1 mg ml or 4 mg/ml. This stimulation is abolished by 1 x 10^{-3} M quinidine. Quinidine at a concentration of 5 x 10^{-4} only partially inhibits the stimulation of glycolysis by 1 mg ml of glucose. Added fructose at a concentration of 5 mg ml produces, unexpectedly, only a slight increase in lactic acid production over the endogenous level. In the presence of added fructose and 1 x 10^{-3} M quinidine, lactic acid production is again reduced to the endogenous level.

Discussion. The experiments reported here show that 1 x 10^{-3} M quinidine inhibits the uptake of added glucose and fructose by rat diaphragm under anaerobic conditions and abolishes the stimulation of glycolysis by glucose and fructose. Endogenous lactic acid production in skeletal muscle has been shown to be at the expense of stored glycogen(7,8). Our observation that quinidine has no effect on endogenous anaerobic glycolysis in intact rat diaphragm suggests that quinidine does not affect any of the metabolic steps between glycogen and lactic acid and therefore inhibits glucose or fructose uptake prior to the step at which these sugars and glycogen share a common metabolic pathway. This suggestion finds support in the work of Uyeki, Geiling and Dubois(3), who found that quinidine did not inhibit lactic acid production from fructose-1, 6-diphosphate in rat heart homogenates.

The inhibitory effect of quinidine on glucose uptake found in the present experiments may account for the decrease in human glucose tolerance noted following quinidine administration(4,5).

Summary. 1. Quinidine sulfate at a concentration of 1 x 10^{-3} M strongly inhibits the uptake of glucose and fructose by intact rat diaphragm under anaerobic conditions. 2. Quinidine is without effect on endogenous anaerobic lactic acid production by rat diaphragm. 3. Glucose at a concentration of 1 mg/ml or 4 mg/ml markedly stimulates anaerobic glycolysis while fructose at a concentration of 5 mg/ml produces only a slight stimulation. 4. Stimulation of glycolysis by glucose or fructose is abolished by 1 x 10^{-3} M quinidine and partially inhibited by 5 x 10^{-4} M quinidine. 5. It is suggested that quinidine inhibits glucose or fructose uptake prior to the step at which these sugars and glycogen share a common metabolic pathway.

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1. Webb, J., Saunders, P., and Nakamura, K., *J. Pharmacol. Exp. Therap.*, 1951, v101, 287.

2. Lee, K. S., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 444.

3. Uyeki, E. M., Geiling, E. M. K., and Dubois, K. P., *Arch. Int. Pharmacodyn.*, 1954, v97, 191.

4. Furman, R. H., and Howard, R. P., *Am. J. Med.*, 1955, v19, 140.

5. Fajans, S. S., Louis, L. H., Sletzer, H. S., Holbrooke, S., Johnson, R. D., and Conn, J. W., *J. Lab. and Clin. Med.*, 1955, v46, 813.

6. Somogyi, M., *J. Biol. Chem.*, 1952, v195, 19; Nelson, N., *ibid.*, 1944, v153, 375.

7. Meyerhof, O., *Arch. Ges. Physiol.*, 1920, v185, 11.

8. Walaas, E., and Walaas, O., *J. Biol. Chem.*, 1952, v195, 367.

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In vitro Effect of Two Pantheine Analogues on Co-Enzyme A Requiring Systems.* (22704)

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The use of metabolic analogues in the study of intermediary metabolism is a method of long standing. That an antagonist of a metabolite active in a multitude of systems would affect each system to varying degrees is predictable in view of the variation in physical constants pertaining to substrate-enzyme, apo-enzyme—co-factor relationships. In the field of vitamin metabolism, experimental data supporting this prediction has only recently been forthcoming(1-4). Analogues have been found which have a very striking antagonizing effect on one or more pathways dependent on the normal metabolite. The same concentration of drug, however, has little effect on other such dependent systems (3,4). These extremes in affinity of metabolic analogues for various systems in which the normal metabolite participates are of great importance in regard to chemotherapy. The present communication extends these observations to analogues affecting pantothenic acid metabolism.

Experimental studies pertaining to the *in vitro* effect of 2 pantethine analogues, omega-methylpantethine and bis (beta-pantoylaminoethyl) disulfide on the ability of pigeon liver homogenates to acetylate sulfanilamide and to synthesize citrate from fumarate and pyruvate are presented in the Table. Although omega-methylpantethine inhibited sulfanilamide acetylation, a similar concentration of this compound was without activity against citrate formation. Increasing the concentration of omega-methylpantethine did, however, have some inhibiting effect on the ability of pigeon liver to synthesize citrate from pyruvate and fumarate. The addition of bis (beta-pantoylaminoethyl) disulfide to an aliquot of the same homogenate inhibited citrate formation. At this same concentration little effect was observed on sulfanilamide acetylation. Increasing the level of bis (beta-

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TABLE I. Effect of Omega-Methylpantethine and Bis (beta-Pantoylaminoethyl) Disulfide on Sulfanilamide Acetylation and Citrate Formation in Tissue Homogenates.*

Treatment		% original activity†	
		Sulfanilamide disappearance‡	Citrate formation‡
None		100	100
Omega-methylpantethine	1.8×10^{-3}M	52	96
	5.5 "	46	86
Bis-(β-pantoyl) disulfide	1.8 "	91	51
	5.5 "	68	15

* All values are avg of 2 separate experiments run in duplicate.

† Control values were 2.68 μM sulfanilamide acetylated/hr/g wet wt in the case of sulfanilamide studies and 28.4 μM citrate formed 50 min./g tissue wet wt in the case of the citrate studies.

‡ Each reaction vessel contained: 250 mg pigeon liver, 0.01 M Mg^{++}, 0.154 M K^+, 0.02 M phosphate, 0.003 M ATP, 4.8×10^{-3}M L-cysteine, 0.03 M acetate, 5.8×10^{-6}M sulfanilamide and pantothenic acid analog as indicated, in a final vol of 2.9 ml. All components were adjusted to pH 7.4.

Each reaction vessel contained 40 mg pigeon liver, 0.01 M Mg^{++}, 0.154 M K^+, 0.02 M phosphate, 0.003 M ATP, 2.4×10^{-3}M L-cysteine, 0.03 M pyruvate, 0.03 M fumarate, 3.5×10^{-3}M sodium monofluoroacetate and pantothenic acid analog as indicated in a final vol of 2.9 ml. All components were adjusted to pH 7.4.

pantoylaminoethyl) disulfide produced inhibition in the acetylation process. Thus, with 2 analogues at the proper concentration, a striking inhibitory effect on one Co-enzyme A dependent system is observed with only slight interference with another Co-enzyme A dependent system.

Co-enzyme A competitively protects transacetylase from inhibition by high concentration of bis (beta-pantoylaminoethyl) disulfide (1). We have confirmed these findings and have extended them by showing that Coenzyme A protects both this system and that concerned with citrate formation against antagonism by either omega-methylpantethine or bis (beta-pantoylaminoethyl) disulfide (unpublished data). Thus, the mechanism of *in vitro* inhibition observed in the above cases, may be attributed to competition between the normal mercaptan, Co-enzymes A, and the related compounds, omega-methylpantethine and bis (beta-pantoylaminoethyl) disulfide.

It must be pointed out that the *in vitro* studies reported probably bear little relationship to the mechanism of carcinostasis observed with both of these compounds(5), since tumor inhibition is obtained with drug concentrations far below the levels used *in vitro*.

Summary. Omega-methylpantethine was observed markedly to inhibit sulfanilamide acetylation at concentrations which were ineffective against citrate formation in pigeon liver homogenates. With aliquots of the same homogenate, bis-(beta-pantoylaminoethyl) disulfide was observed to inhibit citrate formation at concentrations which were ineffective against sulfanilamide acetylation. In either case, higher concentrations of these compounds were effective in inhibiting both systems.

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1. Boxer, G. E., and Shonk, C. E., Stoerk, H. C, Bielimski, T. C., and Budzilovich, T. V., *J. Biol. Chem.*, 1955, v217, 541.

2. Sandman, R., and Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 63.

3. Dietrich, L. S., and Shapiro, D. M., *ibid.*, 1953, v84, 555.

4. Shapiro, D. M., Dietrich, L. S., and Shils, M. E., *Cancer Res.*, 1956, v16, 575.

5. Wolkonsky, P. M., Shapiro, D. M., Shils, M. E., and Dietrich, L. S., *Proc. Am. Assn. Cancer Res.*, 1955, v2, 54.

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Determinants of the So-called "Prothrombin Time".* (22705)

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A recent study(1), assaying the blood-clotting components in normal newborn infants, emphasizes the lack of bleeding disorders and the essentially normal values of the unmodified "prothrombin time" test(2), despite significantly low levels of prothrombin and proconvertin (SPCA, stable factor, factor VII, etc.). It was noted, however, that proaccelerin (AcG, labile factor, factor V, etc.) in the infants' bloods, contrary to previous reports(3,4) was usually *hyper*normal, with values up to 300% of standard normal adults' levels. Dr. Quick(5), and others, have recognized the influence upon the one-stage prothrombin time test of *hypo*normal levels of all 3 of the above thrombin-forming factors. Recently, Quick and Hussey(6) attempted to test effects of adding "excess" of (a) AcG (labile factor) or (b) proconvertin (stable factor) to (1) normal and (2) prothr.-deficient, (3) AcG-deficient, and (4) proconv.-deficient plasmas. They concluded that " . . . a deficiency of any one is *not* compensated by an excess of the other two . . ." (*i.e.* of the 3 thrombin-forming factors). " . . . The determinant of the prothrombin time is the component which decreases below a minimum critical level . . ." As these authors do not give analyses to show the exact amounts of the 3 components in each test, there is considerable question as to the degrees of the deficiencies and "excesses" stated. The following experiments, therefore, were designed to retest various artificial plasma systems, whose assayed composition with reference to the 3 factors under consideration was defined, and could be correlated with the clotting-times after adding calcium and tissue thromboplastin essentially in the manner of the Quick test.

Methods and reagents. Assays: I. (pro) thrombin, (pro) convertin, and (pro) accelerin—the method does not distinguish precursors from the activated forms—are assayed by specific onestage procedures, which follow a common pattern, thus: 0.1 ml of suitable dilution of agent to be tested is mixed with 0.1 ml appropriate "substrate" and the clotting timed at 37°C on adding 0.2 ml *Ca-tpln.* (equal (0.1 ml) vols., preincubated, of 0.02 M $CaCl_2$ and 'Soluplastin', courtesy of Schieffelin & Co.). The *substrates* are modified plasmas lacking the factor to be tested, but complete with regard to the other two(1). Clotting-times are converted into *percentage* units by reference to standard dilution curves obtained with normal adult human plasmas. These "units" (per ml) are employed throughout the present work. Confirmatory prothrombin analyses by the 'improved' 2-stage method(7) are also performed, but give no significant differences in the assay values.

II. *Thrombin* test: 0.2 ml fibrinogen + 0.2 ml agent tested. Observe for clotting.

III. *Antithrombin test*: Incubate 1 ml agent + 1 ml thrombin (bovine, Upjohn's, 20 units/ml) in siliconed tubes, at 25°C, and test 0.2 ml samples with 0.2 ml fibrinogen, at intervals, to observe any loss of thrombic activity.

Materials. Oxalated dog plasma is adsorbed with $BaSO_4$, 100 mg/ml, for 10 min. at 25°C. The supernatant is used to prepare *fibrinogen* by ¼ sat. $(NH_4)_2SO_4$, redissolving the precipitate in ⅓ original vol. of 0.85% NaCl and dialysing against saline containing 0.05 M trisod. citrate. This stock fibrinogen solution is stored frozen at —20°C for many months and is free from prothr., proconv., proaccel., by the above assays.

The $BaSO_4$ sediment is washed (x 2) with dist. water and eluted with ⅓ original (plasma) vol. of 0.2 M trisod. citrate and the *eluate* dialysed against 0.85% NaCl. *Eluate assays. Prep. I.* Prothr. 180, proconv. 100; AcG· trace (±), thrombin 0, antithrombin 0. *Prep. II.* Prothr. 120; proconv. 174; AcG

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TABLE I. Varying AcG, at Several Eluate (Prothrombin + Proconvertin) Dilutions.

Eluate (I)	Pro-thrombin	Procon-vertin	AcG ±	AcG 15	AcG 30	AcG 60	AcG 100	AcG 200	AcG 300
			c.t. (sec.)						
1 : 1	60	33.3	38.2	20.2	18.1	13.7	11.5	*10.6*	*10.2*
1 : 2	30	16.7	48.1	28.1	22.6	16.4	13.9	11.8	*10.4*
1 : 4	15	8.3	64.4	—	23.1	21.6	15.7	14.9	13.7
1 : 8	7.5	4.1	92.	—	—	28.5	23.6	18.5	17.9

±; thr. 0; antithr. 0. *AcG* ('serum'-type or accelerin): $BaCO_3$ adsorbed (4 g/100 ml) beef serum(8), assaying 1240-1380 AcG, and free from proconvertin and all but a very insignificant trace of (pro) thrombin. It contains antithrombin. *Proconvertin* (Prep. A). Serum obtained from recalcified oxalated beef plasma after 18 hrs at 4°C is treated with $BaSO_4$ (10 g/100 ml), the sediment washed (x 4) with dist. water and eluted with 1/5 original vol. of 0.2 M trisod. citrate and the eluate dialysed. This preparation assays 85 *pro*convertin, which special tests show to be mostly in the precursor form, as in the 'prothrombin'-eluates. Other assays: prothr. ±; AcG 0; thr. 0; antithr. ±. Many tests were made with this preparation, but the results were so similar to those with prep. B, in the cited experiments, that they will not be detailed. *Convertin* (Prep. B). Dr. R. H. Wagner *et al.*(9) of our Pathology Dept., kindly supplied us with this preparation, termed (SA) "serum accelerator" in their cited publication in this journal. Our assays show: convertin 81; prothrombin 1; AcG 0; thr. 0; antithr 0.

Test method. Using the above assay values and suitably diluting and mixing the reagents, various *artificial plasmas* are reconstituted. 0.15 ml (*e.g.* 0.05 ml eluate + 0.05 AcG or (pro) conv + 0.05 ml fibrinogen) is tested with 2 vols (*i.e.* 0.3 ml) Ca-tpln., at 37°C, in essentially the manner of the "prothrombin time" test. Such test on 0.15 ml normal dog plasma gives clotting-times of 7-8 seconds.

Results. Table I shows effects of varying the AcG, with 4 different dilutions of eluate (prothr. + proconv.). In each case clotting-times (c.t.) get shorter with increasing AcG. There is a significant failure to find any true "optimum" of AcG concentration. Thus c.t.'s continue to shorten above the 'normal' (100) AcG level even to 3 times that strength. When there is enough prothrombin (and proconvertin), the shortest c.t.'s tests, namely at 300 AcG in the 1:1 and 1:2 dilutions of eluate, are so similar as to be within the experimental error of reading. This is so despite the fact that one has twice the prothrombin (and proconvertin) content of the other. Under these circumstances, the test obviously ceases to be a measure of prothrombin (and proconvertin).

Table II shows effects of varying (pro) convertin (added prep. B), again at 4 different dilutions of eluate. Parallel tests are performed with added excess (300) AcG and with merely the trace (±) of AcG present in the eluates. Clotting-times are shorter, in each series, the higher the (pro) convertin level and the less dilute the prothrombin. The chief conclusion from this experiment is that increasing the (pro)-convertin, up to 85, fails to compensate for lack of AcG or diminishing amounts of prothrombin.

Table III is similar to the foregoing in varying the (pro) convertin and AcG, but the prothrombin is kept at a constant very low level. In each series, there is here a definite small improvement with each incre-

TABLE II. Varying Proconvertin, at Several Eluate Dilutions, with Excess or Trace Amounts of AcG.

Eluate (II)	Pro-thrombin	(Pro)con-vertin	AcG	Clot.-time
1 : 1	40	85	300	8.7"
"	"	"	±	17. "
1 : 2	20	56	300	9.[illegible]"
"	"	"	±	17.8"
1 : 4	10	41.5	300	11.5"
"	"	"	±	22.6"
1 : 8	5	34.3	300	13.4"
"	"	"	±	[illegible]

TABLE III. Varying (Pro)convertin, at Constant Very Low Level Prothrombin (5), and with Excess or Trace Amounts of AcG.

	(Pro)convertin	AcG	Clot.-time
1 a	34.3	300	13.4″
b	34.3	±	28.3″
2 a	20.8	300	14.4″
b	20.8	±	30.6″
3 a	14	300	15.3″
b	14	±	34.2″
4 a	7.3	300	18.2″
b	7.3	±	40. ″

ment of (pro)convertin, in a range of rather low levels. However the large differences due to the major AcG variable persist.

Table IV shows effects of varying (pro) convertin (added prep. B), at constant low prothrombin level, with 4 different levels of AcG. In each case there is a definite inhibitory effect at the highest (69.5) (pro)convertin concentration and least effect at the lowest (29, proconvertin in eluate only), with an apparent optimum (at 56) in the first 3 series. At the highest (200) AcG levels, the effects of varying the (pro)convertin are abolished except for the minor inhibition. The differences between the 4 series which depend upon the AcG level are not significantly altered by varying the proconvertin. In fact, the effects due to the variation of the proconvertin level are quite minor in the range studied.

Table V shows effects of varying (pro)convertin (added prep. B), with constant 'normal' (100) AcG, at 2 fixed concentrations of prothrombin. In 1a the small improvement over 1b (at ½ the (pro)conv. conc.) is real. In 2b the improvement due to increase of (pro)conv. over that in 2c is again significant. In 2a the inhibitory effect of too much added convertin begins to appear (*cf.* Table IV).

TABLE IV. Varying (Pro)convertin, with Low Constant Prothrombin, at Different AcG Levels.

	AcG	Prothrombin	(Pro)convertin* 29	42.5	56	69.5
			c.t.			
1.	±	20	17.0	15.6	15.1	18.5
2.	13.3	20	13.5	11.6	11.2	14.0
3.	66.7	20	10.9	10.0	9.8	10.7
4.	200	20	*9.7*	*9.7*	*9.8*	10.3

* 29 units are *pro*convertin in the eluate, the rest is *convertin* added.

Discussion. Besides confirming the now well established fact that these one-stage clotting times (*cf.* "prothrombin time") depend upon the 3 known variables, prothrombin, AcG, and proconvertin, the present experiments add new information on the relative importance of each and on the results of varying their quantitative interrelationships upon the observed clotting-times.

Most significant is the evidence (Tables I and IV) that in a certain moderately low range of prothrombin (and proconvertin) concentrations a truly large excess, *i.e.* 200-300% of 'normal', AcG can abolish the clotting-time differences, within an experimental error of a few tenths of a second, which would be seen at 100% or lower AcG concentrations and, if shown, be an index of the varying prothrombin (and proconvertin). The AcG level is a very significant variable. On the other hand, the (pro)convertin, at any fixed prothrombin or AcG level, exerts relatively minor effects although these are not insignificant. Increasing amounts of (pro)convertin do compensate somewhat for reduced prothrombin levels, but very incompletely and they do not at all abolish the large differences due to varying AcG. There is an experimental limit to our tests on these points due to the appearance of an inhibitory phenomenon. This could not be attributed to antithrombin, since it was not seen with excessive amounts of the AcG preparation which was rich in that factor. It was seen even more in the non-cited experiments with our own *pro*convertin (prep. A), which did have a small amount of antithrombin. The antithrombin test with Dr. Wagner's convertin, however, was negative,

TABLE V. Varying (Pro)convertin, with Constant Normal (100) AcG, and 2 Fixed Concentrations of Prothrombin.

	AcG	Prothrombin	(Pro)convertin*	c.t.
1 a	100	40	94.8	9.3″
b	100	40	58	9.9″
2 a	100	20	80.3	10.9″
b	100	20	51.3	10.1″
c	100	20	29	12.0″

* In 1 series, 58 units are *pro*convertin in eluate; in 2 series, 29 units are *pro*convertin in eluate.

but it, too, gave inhibition (see Tables). Whether these inhibitory effects and the apparent "optimum," for concentrations of (pro)convertin, are due to (a) some unidentified inhibitor contaminating the present preparations, or (b) some type of competitive reaction in the complex sequence of the thrombin-forming processes, are matters which will require further investigation.

Further technical questions may arise concerning possible difference in one-stage systems, between *pro*-accelerin or *pro*convertin and their 'activated' forms, accelerin or convertin, respectively. Nothing in our test system suggests such a possibility and the determinants we have sought by adding the 'activated' forms, in all probability, hold also for their precursors. We believe, therefore, it is reasonable to transfer the results of these experiments to interpretations of "prothrombin times" encountered in ordinary plasmas. In particular, they offer an attractive explanation of the frequently normal "Quick" test on newborn infants' blood, namely, that the very considerable *hyper*-proaccelerinemia of such cases(1) can *compensate* for the normally low levels, in infants, of prothrombin and proconvertin. There is no need, in our opinion, to postulate any obscure "convertibility" factor or some hypothetical "prothrombinogen."

Summary. In analysed systems representing artificially reconstituted plasmas of known composition with respect to prothrombin, (pro)convertin, and AcG, one-stage clotting-times with Ca-thromboplastin, essentially in the manner of the "prothrombin time" (Quick) test, show: (1) prothrombin concentration is a major variable. (2) (pro)convertin concentration is a minor variable, which, except at very low(10) levels, affects the clotting-times significantly only at very low prothrombin levels. (3) High (pro)convertin levels, short of an unexplained inhibitory phenomenon (minor), do not compensate significantly for low levels of prothrombin or of AcG. (4) AcG levels are a major variable. (5) Excessively high (200-300% of 'normal') AcG levels *can compensate* for moderately low prothrombin and proconvertin, causing the one-stage test to lose significance as a measure of these other two factors. (6) It seems valid to apply this last result to explain the normal "prothrombin time" (and lack of bleeding) in normal healthy newborn infants(1).

1. Fresh, J. W., Ferguson, J. H., and Lewis, J. H., *Obst. and Gyn.*, 1956, v7, 117.

2. Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. Med. Sci.*, 1935, v190, 501.

3. van Creveld, S., Paulssen, M. M. P., and Teng, S. K., *Neonatal Studies*, (Amsterdam), 1952, v1, 1.

4. Larrieu, M. J., Soulier, J. P., and Minkowski, A., *Centre internat. de l'enfance.* Étud. néo.-nat., 1952, v1, 1.

5. Quick, A. J., *The physiology and pathology of hemostasis.* (Lea & Febiger, Phila.), 1951, p188.

6. Quick, A. J., and Hussey, C. V., *Brit. Med. J.*, 1955, (1), 934.

7. Ferguson, J. H., Lewis, J. H., and Fresh, J. W., *Yale J. Biol. and Med.*, 1956, v28, 253.

8. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

9. Wagner, R. H., Brannan, W. M., Jr., Brinkhous, K. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 266.

10. Lewis, J. H., Fresh, J. W., and Ferguson, J. H., *ibid.*, 1953, v84, 651.

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Age Changes in Composition of Aorta of the Rabbit.* (22706)

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The important disabling and lethal vascular changes of ageing in man include those of atherosclerosis, calcification and hypertension. There has been considerable research, in man and in animals, on dietary induction of these conditions, and on alterations in blood chemistry that accompany their development (1). There are also reports on chemical alterations in vessels affected by atherosclerosis (2), calcification(3) and hypertension(4). However, there has been relatively little reference to age changes in chemical composition of vessels *not* affected by one or more of these conditions. Study of this aspect of vascular disease seems important, since the vessels of the young are relatively free of these damaging conditions. The present study was undertaken to determine some of the chemical phenomena of vascular ageing in the hope that the results might indicate some local factors that might render the vessels of the old more susceptible, if indeed they are, than those of the young. In view also of the rarity in ageing rabbits of spontaneous vascular disease equivalent to that in man, it was hoped that comparison of the findings in these two species might yield information bearing on the pathogenesis of the diseases noted above. It has been reported that in man the water content of the aortic wall either remains constant(3,5) or rises slightly(6) with increasing age. This is accompanied by progressive elevation of the total lipid(3,5) and of cholesterol(2,7). Calcium(3,5,8,9) and phosphorus (3) have also been reported as reaching higher concentrations in the human aorta with advancing age. Data on the sodium and potassium content of the human aorta have not come to our notice.

There appear to be no comparable data on age changes in the chemical composition of the rabbit aorta. Our preliminary studies showed that in ageing rabbits there is a significant and progressive rise in the water content of the aorta, associated with an even more striking fall in the quantity of combined aqueous and fat-soluble extractibles, (unpublished data). The present study was undertaken to confirm and amplify this observation.

Materials and methods. Forty-four female and 7 male New Zealand white rabbits were used. Because an adequate number of old males could not be obtained, the results tabulated and tested statistically are for the females only. There were 27 young animals, aged 2.5 to 3.5 months, and 24 old ones of 37 to 55 months. All were apparently healthy. Samples of blood were collected from a few animals (via the ear artery) several weeks before killing, and also from some immediately before killing. Some were subjected to blood pressure determinations by the non-traumatic method of Grant and Rothschild (10). All were fed Ogilvie Miracle Rabbit Pellets® and tap water *ad libitum*. The average period of acclimatization in our animal quarters after delivery from the dealer was one month. The environment was at normal indoor or "room temperature." The animals were killed by a blow on the back of the neck, and autopsies done immediately. The aorta from arch to renal arteries was removed, opened longitudinally, blotted free of gross blood with filter paper, and the adventitial fat dissected off as completely as possible. The specimen was transferred to a stoppered weighing bottle, placed in a desiccator, and weighed to 0.1 mg. The tissue was then lyophilized and the dry weight obtained. In transfer procedures the aorta was handled with forceps only. Sodium, potassium and calcium concentrations were determined using aortas from 18 young rabbits (all female) and 16 old ones (12 females, 4 males). Samples were pooled in 3's from young, and in pairs

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TABLE I. Age Changes in Aortic Water of Rabbit.

No. of animals	Avg age, mo	Water, %
25	3	62.9 ± .4
19	46	65.4 ± .3

from old animals, to provide quantities large enough for accurate measurement. The pooled samples were ashed with nitric and perchloric acids. Sodium and potassium were determined by flame photometry, and calcium by the microadaptation of the Clark-Collip method(11). Data from female samples are designated in Table II as Group 1-a and 2-a. The aortas of the remaining 9 young (7 females, 2 males) and 8 old (7 female, 1 male) rabbits were used for determinations of total lipid, cholesterol and non-lipid phosphorus. These vessels were also tested for carbohydrates, reducing sugars and sulfur. Data from females are listed as groups 1-b and 2-b, Table III. After freeze-drying, the specimens were extracted in a Soxhlet apparatus, using 15 cc ethanol for a period of 24 hours, followed by cold ethyl ether overnight. The fat-free weight of the sample was recorded and the aorta was extracted in 15 cc double distilled water for an additional 24 hours. Following this the sample was desiccator-dried and weighed. The alcohol-ether extracts were evaporated and the residue taken up in petroleum ether. Total cholesterol content of this sample was then estimated by the Liebermann-Burchard reaction (12). The water extract was combined with the residue remaining after purification of the alcohol-ether extract and was tested for traces of carbohydrate, reducing sugars and sulfur. Molisch's reagent was used for carbohydrate, Benedict's quantitative reagent for reducing sugars and barium chloride solution for sulfur(13). The phosphorus content of the aqueous extract was estimated on duplicate aliquots using the Fiske-Subbarow technic (14).

Results. The qualitative tests for carbohydrate, reducing sugars and sulfur yielded negative results. Table I shows the mean values for water content of the aortas of 25 young and 19 older female animals. The aortas of the older animals contain an average of 2.5% more water than those of young animals, a small but highly significant difference ($p<.001$). Because of this change in water concentration, other values are reported per unit of dry weight.

In Table II are shown the aortic sodium, potassium and calcium concentrations. The potassium content remains unchanged, while there is a moderate but significant increment of sodium, and the calcium concentration is approximately doubled in the older animals ($p<.001$ for both Na and Ca). These changes are more marked than the increase in water concentration, and are significant even when related to the wet weight.

Table III compares the aortic content of lipid, cholesterol and phosphorus in the young and old animals. The results indicate a slightly but not significantly lower total lipid, and moderately higher levels of cholesterol ($p<.05$) and of phosphorus ($p<.02$) in the aortas of the older animals.

Discussion. These results confirm our pre-

TABLE II. Age Changes in Sodium, Potassium and Calcium of Aorta of Rabbit.

Group	No. of pooled samples	Age, mo	Na	K	Ca
			(mEq/100 g dry wt)		
1-a	6 (18)*	3	22.4 ± .4	11.3 ± .4	98 ± 9
2-a	6 (12)	46	28.6 ± 1.2	11.5 ± .3	219 ± 15

* Figures in parentheses show No. of aortas used.

TABLE III. Age Changes in Lipid, Cholesterol and Non-Lipid Phosphorus of Aorta of Rabbit.

Groups	No. of animals	Age, mo	Lipid, % dry wt	Cholesterol	Non-lipid phosphorus
				mg/100 g dry wt	
1-b	7	3	16.3 ± 1.7	463 ± 37	158 ± 12
2-b	7	45	13.7 ± 1.3	558 ± 22	210 ± 15

liminary finding of a higher aortic water concentration in old than in young rabbits although the increase found in this study, using freeze-drying, is less marked than that found previously by oven-drying at 110°C. Comparable studies in man have shown that aortic water either remains constant(3,5) with increasing age, or increases(6).

The rise in water content of the aorta associated with a rise in the sodium but constant potassium level might indicate that all or most of the added water was extracellular, *i.e.* that the aged aorta had become relatively oedematous. While this may well be so, there is some evidence to indicate that the change may be more complex. Other studies(15) have indicated age changes in the concentrations of sodium and potassium in rabbit erythrocytes, characterized by progressive replacement of intracellular potassium by sodium. If the same should be true of the cells of the aortic wall, then the extra water and sodium in the aortas of old animals could be both intra- and extracellular. Elevation of aortic water, sodium and potassium was found by Tobian and Binion(16) in hypertensive rats. The relation of the sodium increase to blood pressure in our animals is not clear. In general, adult rabbits have shown higher pressures than animals comparable in age to those of the present young group. However, other rabbits with a marked elevation of blood pressure due to bilateral renal compression did not have significantly altered aortic water, sodium or potassium concentration. A similar discrepancy was found for red cell electrolytes(15).

In the case of the other mineral constituents studied, calcium and phosphorus, our findings are in general agreement with those reported by others in man(3,5,8,9). In spite of the fact that in none of the aortas examined was there the slightest evidence of gross calcification, analysis showed a 100% increase of calcium in the older aortas. Phosphorus was increased by slightly more than 20%. The increment of phosphorus in the older animals is in about the same ratio to that of calcium as was found in man by Buck (3), *i.e.* 0.36:1. The calcium and phosphorus concentrations in the rabbit aorta were both considerably lower than Buck's lowest human values. This is curious because serum calcium levels in the rabbit are normally nearly double those in man(17).

A further difference between age changes in chemical constituents in the rabbit aorta and those in man is seen in the concentrations of total lipid and of cholesterol. For both these constituents the concentrations in the rabbit aorta are much lower than those reported in man(3,5,7). In addition, while the concentration of total lipid rises progressively with advancing age in man, our findings in the rabbit indicate no such increase, though there is an increase of doubtful significance ($p<.05$) in aortic cholesterol. It is worth noting that the only old male whose aortic cholesterol and total lipid were determined had values for both which were lower than those of any of the 7 old females studied.

In the interpretation of any age alterations in the rabbit one is handicapped by the lack of precise data on the natural longevity of rabbits as noted by Leary(18). On the basis of available information we have chosen to consider the two groups of animals in the present study as representing approximately childhood and middle-age.

Summary. A study of the aortas of 44 female rabbits in 2 age groups (3 mos. and 46 mos.) corresponding approximately to human childhood and middleage, indicates elevation of the water, sodium, calcium, non-lipid phosphorus, and possibly of the cholesterol content of the vessel wall in the older group. The potassium content remains relatively constant. The generally lower levels of total lipid and of cholesterol in the rabbit aorta compared with that of man probably account at least in part for the freedom of the former from spontaneous atherosclerosis.

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1. Duff, G. L., and McMillan, G. C., *Am. J. Med.*, 1951, v11, 92.

2. Buck, R. C., and Rossiter, R. J., *A.M.A. Arch. Path.*, 1951, v51, 224.

3. Buck, R. C., *ibid.*, 1951, v51, 319.

4. Weigensberg, B. I., Ph. D. Thesis, McGill University, 1953.

5. Weinhouse, S., and Hirsch, E. F., *A.M.A. Arch. Path.*, 1940, v29, 31.

6. Myers, V. C., and Lang, S. W., *J. Gerontol.*, 1946, v1, 441.

7. Faber, M., *Acta Med. Scand.*, 1948, v125, 418.

8. Lansing, A. I., Alex, M., and Rosenthal, T. B., *J. Gerontol.*, 1950, v5, 314.

9. Miller, H., Hirschman, A., and Kraemer, D. M., *A.M.A. Arch. Path.*, 1953, v56, 607.

10. Grant, R. T., and Rothschild, P., *J. Physiol.*, 1934, v81, 265.

11. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, v63, 461.

12. Notes on Operation of the Evelyn Colorimeter. The Rubicon Co., 1945.

13. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, 12th Ed. Blakiston Co., Toronto, 1947.

14. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

15. Waugh, D., and Stuart, J. R., *Am. J. Path.*, 1955, v31, 575.

16. Tobian, L., Jr., and Binion, J. T., *J. Clin. Invest.*, 1954, v33, 1407.

17. Popovici, A., Geschickter, C. F., Reinovsky, A., and Rubin, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 415.

18. Leary, T., *Arch. Path.*, 1934, v17, 455.

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Serial Propagation of 3 Strains of Rabbit Fibroblasts; Their Susceptibility to Infection with Vaccinia Virus.* (22707)

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Tissue culture technics have been employed frequently for the propagation of viruses *in vitro*(1,2). There have been, however relatively few studies that attempt to elucidate in a simplified system which is potentially provided by tissue culture many fundamental problems regarding virus multiplication. To approach problems of this type in a definitive manner, it is necessary to have a continuous supply of uniform experimental material such as is provided by cultures of mammalian cells which can be propagated serially under controlled conditions. Cultures derived from different sources may vary considerably in their viral range and in their response to viral infection in a fashion analogous to laboratory animals. This indicates the need for cultures of more than one cell type as well as cells derived from a variety of animals. These studies were undertaken to investigate the possibility of establishing *in vitro*, strains of fibroblasts derived from rabbit tissues and to compare their relative susceptibilities to infection with vaccinia virus.

TABLE I. Composition of Cell Culture Media.

	Composition* (V/V)		
Medium	%	%	%
CF	5 CEE	10 NHS	85 V-614
F7		10	90
F15	5 BEE	10	85
F17	10 CEE	20	70
F21	5	20	75
705	5	20	75 703

* The following abbreviations are employed: CEE = chick embryo extract; BEE = beef embryo extract; NHS = normal horse serum; V-614 = solution of Fischer *et al.*(5); 703 = solution 703 of Healy *et al.*(6).

Materials and methods. Media. The composition of the media employed for propagating cells is indicated in Table I. All media were sterilized by filtration through Selas filters of 02 porosity. Chick embryo extract (CEE) and beef embryo extract (BEE) were prepared as described previously(3). *Establishing cell cultures on glass.* Cell cultures were initiated in roller tubes according to the method of Swim and Parker(4). When growth in the roller tubes was well established, the cells were dislodged from the glass by treating with 0.02% trypsin (Nutritional Biochemicals Corp., 2 x crystallized—50% Mg SO_4) in V-614 solution(5) or in solution 703 (6), pH 7.9-8.3, for 10 minutes at 37°C. The cells were then washed in nutrient fluid, the cell clumps largely dispersed by repeated pipetting. The cells were counted in a hemocytometer and 1.5×10^6 were added to T-60 flasks(7). The medium in each flask was replaced 3 times weekly. Subcultures were prepared by the use of trypsin. After treatment with trypsin, the cells were washed in nutrient fluid and added to fresh flasks (8×10^5 cells per T-60). *Vaccinia virus.* The CVII strain of vaccinia virus(8) which had been passed 1 to 3 times in cultures of rabbit-muscle fibroblasts was employed. *Titration of vaccinia virus in cell cultures.* One ml aliquots of a suspension containing 5×10^4 cells were added to 13 x 100 mm Pyrex tubes. Serial 10 fold dilutions of virus were prepared in solution V-614 and 0.1 ml of each dilution was added to a series of 5 tubes. The tubes were inclined at an angle to 5° and incubated at 37°C in a stationary position. After 24 hours, the tubes were transferred to a roller drum at 37°C. The virus titer ($TC\text{-}ID_{50}$) was determined on the basis of the lowest concentration of virus which destroyed the cells in 9 days by the method of Reed and Muench(9).

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* Aided by a grant from The National Foundation for Infantile Paralysis.

† U. S. Public Health Service Research Fellow of The National Microbiological Institute.

Results. Twenty-three strains of fibroblasts derived from tissues of the rabbit, and chick embryo were established *in vitro* and propagated serially in a variety of media. In spite of concentrated efforts to establish the conditions required for the continuous propagation of each strain of cells, only 3 proved to be capable of sustained growth in the media employed. The pedigrees of the successful strains are outlined in Fig. 1. It will be noted that the growth rate of each strain progressively declined after a period of active proliferation. This was accompanied at first by an increase in the number of granules in the cytoplasm of the cells; later, degenerating cells were observed and their numbers increased progressively until the bottoms of the flasks were covered with a dense layer of cellular debris (Fig. 2). Some of the cells continued to metabolize glucose, however, as indicated by the production of acid and fluid replacements were continued at regular intervals. During the 19th week a colony (about 1 mm in diameter) of cells, of normal appearance (Fig. 3), was observed in one of the four flasks in the series derived from adult rabbit testicle. The progeny of the cells from this colony (strain RT6-F17; Fig. 4) have continued to proliferate at a constant rate and are currently in the 57th passage.

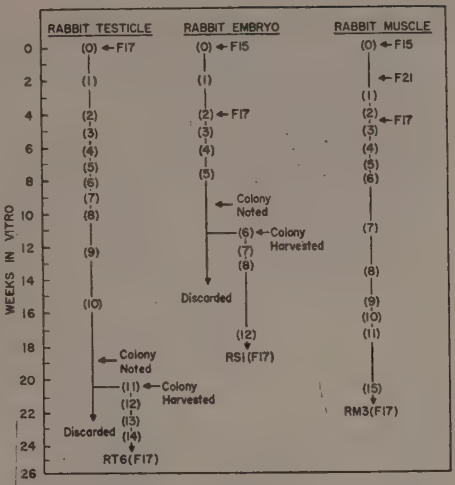
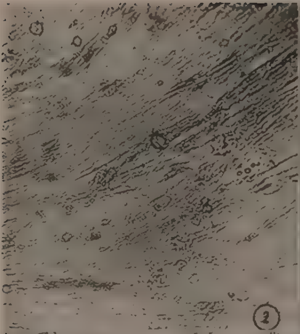


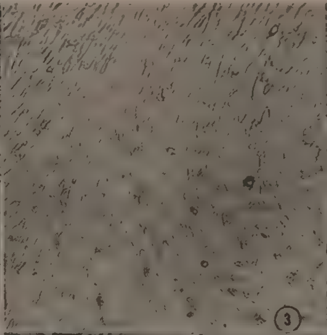
FIG. 1. Pedigrees of 3 strains of rabbit fibroblasts. Figures in parentheses refer to subculture number.

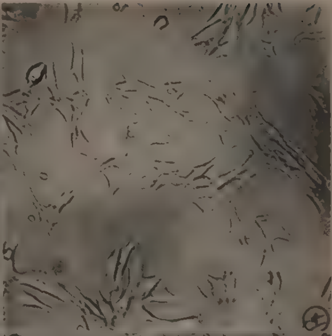
A second strain of fibroblasts was derived from the leg of a rabbit embryo of approximately 15 days' gestation (Fig. 1). During the 10th week three isolated colonies of normal appearing cells were observed in two of the five flasks in this series. The progeny of the cells from one of these colonies (Strain RS1-F17; Fig. 5) are now in the 38th passage.

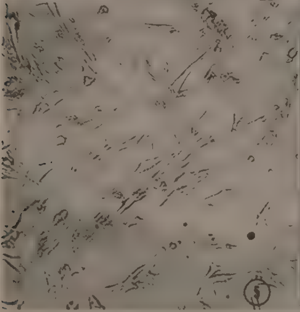
A third strain of fibroblasts was derived from adult rabbit skeletal muscle. In this instance the degenerative changes which accompanied the decline in growth rate (Fig. 1) were not as extensive as those observed with strain RT6-F17 and RS1-F17. During the 13th week the overall appearance and growth rate of the culture began to improve gradually and after the 16th week it was possible to prepare subcultures at weekly intervals. This strain of fibroblasts, (RM3-F17; Fig. 6), continues to proliferate rapidly after 61 passages.

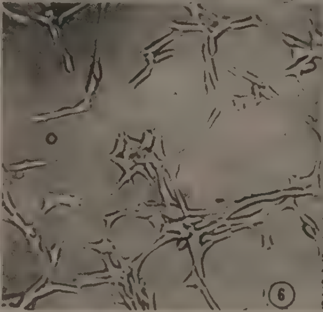
The other 20 cultures of fibroblasts which











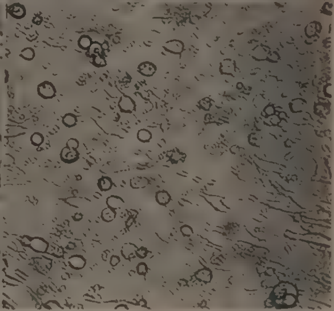


FIG. 2. Culture derived from rabbit testicle after 18 wk *in vitro*.
FIG. 3. Fibroblasts in original colony of RT6-F17 derived from culture illustrated in Fig. 2.
FIG. 4. Rabbit fibroblasts, strain RT6-F17 during 57th subculture.
FIG. 5. Rabbit fibroblasts, strain RS1-F17.
FIG. 6. Rabbit fibroblasts, strain RM3-F17.
FIG. 7. Culture of RM3-F17 three days after infection with 100 TC-ID$_{50}$ of vaccinia virus.

were established (5 rabbit testicle; 2 each rabbit muscle, kidney, liver and lung; 7 chick-embryo heart or skeletal muscle) proliferated rapidly for 8-12 subcultures in a variety of media in addition to those indicated in Table I. After this period, the rate of growth either declined gradually over a period of several weeks or growth ceased abruptly. Numerous attempts to formulate a medium that would prolong the period of active growth of these cells have been unsuccessful. Each strain was maintained by periodic replacement of the medium until no viable or metabolizing cells appeared to be present in the flasks. Several cultures of chick embryo fibroblasts were maintained for 9 months without subculturing but failed to yield cells capable of continuous proliferation.

The following is a summary of the general nutritional requirements of the 3 strains of fibroblasts. Solution V-614 supplemented with CEE extract and NHS (medium F17, Table I) is an excellent medium for all 3 strains of fibroblasts. Media containing less NHS and CEE than F17 are adequate but the growth rate of cells is decreased proportionately whereas, media containing higher concentrations of NHS and CEE than F17 are inhibitory. The medium formulated by Eagle(10) for strain L will replace solution V-614 in F17 medium but the cells fail to proliferate when solution 199(11) or 703(6) is used. CEE is superior to BEE and extracts prepared from various adult rabbit organs are toxic. NHS can be replaced by bovine serum whereas human or rabbit serum is unsatisfactory.

Vaccinia virus multiplies in each of the 3 strains of fibroblasts and this is accompanied by the destruction of cells (Fig. 7). This cytopathogenic action afforded a method for comparing the susceptibility of cell strains to

TABLE II. Relative Susceptibilities of Cell Cultures to Infection with Vaccinia Virus.

Culture	Negative logarithm TC-ID$_{50}$/ml CF*	F15*	F7*
Lymphosarcoma (MB13)[†]	6.73	6.50	[‡]
Chick embryo[§]	6.08	5.50	[‡]
RM3 (F17)	5.82	5.12	4.68
RT6 "	5.55	5.55	4.84
RS1 "	5.50	5.38	4.44

* See Table I for composition of media; each value is avg for 4 or more titrations.

[†] Serially propagated in medium 705 as described previously(3).

[‡] Cells degenerated in absence of virus.

[§] Fibroblasts derived from skin-muscle of 10-day embryo; serially cultivated in CF; titrations performed at intervals from 3rd to 12th subculture.

vaccinia by employing them for titration of a standard pool of virus.

The relative susceptibilities of the 3 strains of rabbit fibroblasts are presented in Table II, together with results obtained with chick embryo fibroblasts and with lymphosarcoma cells, strain MB13. Strains RM3-F17, RT6-F17, RS1-F17 and chick embryo fibroblasts exhibit the same degree of susceptibility (medium CF) which is less than that observed for MB13 by a factor of approximately ten. The titer observed with MB13 is the same as that obtained in the embryonated egg by the pock method(12). When BEE is substituted for CEE in the medium, the relative susceptibilities of RT6-F17 and RS1-F17 are unchanged whereas, RM3-F17 appears to be less susceptible (compare media CF and F15). On the other hand, when embryo extract is omitted (medium F7) the 3 strains of rabbit cells are uniformly less susceptible. It is of interest that the susceptibility of RT6-F17 (subcultures 13-15; Fig. 1) which was derived from an isolated colony of cells is the same as the parent culture from which it was derived (subcultures 3-6; Fig. 1).

Discussion. The fact that all of the cells derived from normal tissue either ceased to proliferate or multiplied at a sharply reduced rate after an initial period of luxuriant growth is a perplexing problem which is not confined to fibroblasts from rabbit and chick embryo tissues[‡](13). This phenomenon has received a minimum of attention primarily because in most instances cultures derived from normal tissues have not been propagated on glass for sufficient periods to observe this phenomenon. For example, certain strains of human fibroblasts cease to proliferate only after 30 subcultures *in vitro*.[‡] One interpretation of the results of this study is that the usual tissue culture media are inadequate because they lack essential nutrients or because they are chronically toxic to most fibroblasts.

The results of this survey raise the question of the origin of the fibroblasts capable of continuous proliferation since only 3 of the 23 strains examined yielded such cells and because the latter appeared at a time when most of the cells in the individual cultures were either dead or degenerating. These data are subject to at least 2 interpretations: (1) fibroblasts capable of continuous proliferation were present in the original tissue inoculum and were preferentially selected by serial propagation; (2) in the course of serial propagation, a small proportion of the cells were altered sufficiently by mutation to be capable of sustained growth in F17. The feasibility of the latter is supported by studies of Sanford *et al.*(14) and by the fact that strains of human fibroblasts have been observed to alter their nutritional requirements on prolonged serial cultivation in response to changes in the composition of the medium.[‡] Regardless of the mechanism with regard to the origin of the 3 strains of rabbit fibroblasts, it is clear that the proportion of cells derived from tissues of the rabbit and the chick embryo which are capable of sustained growth in the media employed is a very small fraction of the total. Furthermore, it appears that the isolation of permanent lines of fibroblasts from these sources as a routine procedure will require modifications in the composition of the usual tissue culture media.

Morphologically all 3 strains of rabbit fibroblasts are similar and each has maintained its characteristic appearance throughout the period *in vitro*. The most striking difference observed between strains RM3-F17, RT6-F17 and RS1-F17 is that the rate of growth of the former in F17 (12 fold per week) is about twice that of the other 2 strains (Table II).

[‡] Unpublished experiments.

Summary. Twenty-three strains of fibroblasts derived from normal rabbit and chick embryo tissue were serially propagated on glass in a variety of media. All except 3 strains failed to proliferate for more than 8 to 12 subcultures. The 3 successful strains were derived from adult testis, embryonic skin-muscle and adult muscle respectively and continue to proliferate at a constant rate after 38 to 61 subcultures. Vaccinia virus multiplies in all 3 strains of fibroblasts resulting in the complete destruction of cells.

1. Robbins, F. C., and Enders, J. F., *Am. J. Med. Sci.*, 1950, v220, 316.
2. Sanders, M., *Arch. Path.*, 1953, v56, 148.
3. Swim, H. E., and Parker, R. F., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 549.
4. ———, *ibid.*, 1953, v83, 577.
5. Fischer, A., Astrup, T., Ehrensvard, G., and Oehlenschlager, V., *ibid.*, 1948, v67, 40.
6. Healy, G. M., Fisher, D. C., and Parker, R. C., *Can. J. Biochem. Physiol.*, 1954, v32, 327.
7. Earle, W. R., and Highouse, F., *J. Nat. Cancer Inst.*, 1954, v14, 841.
8. Rivers, T. M., and Ward, S. M., *J. Exp. Med.*, 1933, v58, 635.
9. Reed, L. J., and Meunch, H., *Am. J. Hyg.*, 1938, v27, 493.
10. Eagle, H., *Science*, 1955, v122, 501.
11. Morgan, J. F., Morton, H. J., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 1.
12. Burnet, F. M., *J. Exp. Med.*, 1936, v63, 685.
13. Perry, V. P., Evans, V. J., Earle, W. R., Hyatt, G. W., and Bedell, W. C., *Am. J. Hyg.*, 1956, v63, 52.
14. Sanford, K. K., Likely, G. D., and Earle, W. R., *J. Nat. Cancer Inst.*, 1954, v15, 215.

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Studies on Utilization of Sulfate Sulfur for Growth of the Chicken.[*][†]

(22708)

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It has been reported(1) that addition of sodium sulfate to a purified diet devoid of inorganic sulfur improved growth and feed efficiency of young chickens. Studies with S^{35}-sulfate have demonstrated that small amounts of sulfate sulfur are incorporated into cystine in the growing chicken(1,2) and laying hen (3), and larger portions into taurine by the chick embryo(4). In addition, the feathers of birds fed $Na_2S^{35}O_4$ contain a radioactive organic fraction which is not a sulfur amino acid and which has not been identified(1).

The purpose of these studies was to verify the growth response from sulfate sulfur, to determine whether such a response results from the conversion of sulfate sulfur to sulfur amino acids and to identify the radioactive compound appearing in the feathers after the feeding of $Na_2S^{35}O_4$.

Growth. The basal diet was a modification[§] of that used by Gordon and Sizer(1), which contains 15% casein and 10% gelatin as the protein[||] source. Female New Hampshire X Silver Cornish crossbred chicks were fed the basal diet for 1 week. They were then weighed, divided into groups of 13 chicks of equal weight distribution and fed the experimental diets for 3 weeks. The chicks were reared under electrically heated brooders in wire-floored cages, in an air-conditioned room. Addition of 0.5% Na_2SO_4 to the basal

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[†] An abstract of part of this work was published in *Poultry Sci.*, 1955, v34, 1209.

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[§] Wesson oil or lard was used as the fat. $MgCO_3$, $MnCl_2$ and copper acetate were used instead of the corresponding sulfates.

[||] The protein furnished about 0.54% methionine and 0.06% cystine. Since the estimate corresponds closely to the 0.132% total sulfur found by analysis the diet probably contained no inorganic sulfur.

TABLE I. Growth Response from Addition of 0.5% Na$_2$SO$_4$ to Low-Sulfur Diet.

Exp. No.	Basal diet	0.5% Na$_2$SO$_4$	Increase, %	Level of significance*
	Body wt (g) at 4 wk			
1	160	232	45	.01
2	157	174	11	none
3	192	237	23	.01
	175	210	20	.05
4	285	300	5	none
5	204	283	39	.01
	224	276	23	.01
6	228	274	20	.05
7	239	294	23	.05
8	230	262	14	none
9	245	284	16	.05
Avg	213	257	22	

* Probability using Students' "t" test.

diet resulted in a growth response in every trial which was usually statistically significant (Table I). Feathering of birds fed the basal diet appeared normal.

Utilization of $Na_2S^{35}O_4$. A group of chicks was fed the basal diet plus 0.4% DL methionine. At 2 weeks of age the chicks were fed this diet plus 5 mg of Na$_2$S^{35}O$_4$ (1.6 mc) per kilo. One and 2 weeks after the addition of radioactive sulfate, 5 chicks were killed and the S^{35} in the sulfate, cystine, methionine, and taurine fractions determined for individual carcasses. The methods were essentially those previously described(5), except that paper chromatography was employed for further purification of taurine following chromatography on Dowex-50 resin. A summary of these results is given in Table II. Only trace amounts of radioactive cystine or methionine could be detected at 1 week following feeding of radioactive sulfate. After 2 weeks there was a measurable amount of radioactivity in these amino acids. Since only 1.5% of the S^{35} ingested was recovered in the cystine and methionine it would not appear that the rate of incorporation of sulfur into these amino acids was sufficient to significantly reduce the requirement for these amino acids in the diet. Taurine-S^{35} accounted for about 19% of the S^{35} recovered in the birds after 1 week of sulfate-S^{35} feeding and 24% after 2 weeks. This increase would suggest that taurine has a longer biological half-life than sulfate. A portion of the organic-S^{35} could not be accounted for as either cystine, methionine or taurine. It is likely that some of this S^{35} was present as unidentified compounds. These probably arise from the metabolism of taurine (6). Taurine accounted for an average of 6.7% and sulfate 5.6% of the total sulfur of the carcass of 4-week-old chickens.

TABLE II. Recovery of S^{35} from Compounds Isolated from Carcass of Chickens Fed Na$_2$S^{35}O$_4$.*

Compound	Duration of feeding period (wk)† 1	2
	% of total S^{35} recovered	
Sulfate	74.5	57.8
Cystine	trace	1.4
Methionine	"	.1
Taurine	18.9	24.1
Remainder	6.6	16.6
Total	100.0	100.0
% of dose‡ administered	22.7	19.7

* 1.6 mc/g of diet.

† Chickens were 2 wk of age at start of radioactive feeding period.

‡ Based on avg feed consumed.

Feather compound. The feathers from the birds fed sulfate-S^{35} were analyzed after acid hydrolysis. Sulfate, cystine and methionine accounted for only 47% of the S^{35} in these feathers. The remainder of the S^{35} behaved similarly to taurine on Dowex-50 columns. After paper chromatography, however, it did not give the same R$_f$ as taurine with all solvent systems. A brown pigment was present in fractions containing the unknown S^{35} compound. In order to avoid any complication from this pigment in the colored feathers of the chicks used, a second study was conducted with White Leghorn chicks. In this study it was found that almost all of the unknown S^{35} could be extracted from the ground feathers with hot water. This water extract was subjected to chromatography on Dowex-50(4), followed by paper chromatography with 5 different solvent systems. Essentially all of the non-sulfate-S^{35} in the extract was thereby identified as taurine-S^{35}. Approximately 54 mg% of taurine (on dry matter basis) was found(4) in these feathers after acid hydrolysis. This accounted for about 10% of the free amino nitrogen and 0.6% of the total sulfur in the feathers.

A third study was conducted with New Hampshire X Silver Cornish birds. The S^{35} compound was freed of contamination with pigment by eluting it from large sheets of Whatman 3MM filter paper after development of radioactive fractions with n-propanol-1N NH_4OH (5:1). When this unknown S^{35} was rechromatogramed 90% of the S^{35} was found at the R_f values corresponding to taurine and the R_f was not changed after treatment with H_2O_2. With some chromatograms a small second peak of S^{35} was detected suggesting the presence of another sulfur compound. However, most of the unknown S^{35} was apparently taurine whose detection was obscured by the presence of feather pigment.

The significance of taurine in feathers is not known. It has been suggested(6) that end-products of metabolism such as uric acid and creatinine are "trapped" in the feather during the process of rapid growth and keratinization. Similar reasoning could be applied to taurine.

Discussion. Our work confirms the report of Gordon and Sizer(1) on the ability of sodium sulfate to stimulate the growth of the chicken, but differs with their observations on feathering. These workers reported that feathering of chickens fed a low-sulfur diet was poor and was improved by addition of sulfate. In our experience, the feather development of chickens fed a low-sulfur diet was satisfactory and showed no improvement when sulfate was added to the diet. Sulfate sulfur was not utilized for synthesis of sulfur amino acids in amounts large enough to suggest nutritional significance, but was incorporated into tissues chiefly as protein-bound sulfate. The growth response from feeding sulfate therefore may be due to a physiological requirement for sulfate *per se*. This requirement can evidently be satisfied by dietary sulfate or by sulfate furnished by the oxidation of the sulfur amino acids. Thus sulfate may "spare" the methionine or cystine which would be oxidized to provide the sulfate necessary for the growth of the chicken. The sulfur amino acid requirements have been determined with basal diets containing varying amounts of sulfate sulfur. Therefore, on the basis of data now available, it is impossible to estimate to what extent sulfate sulfur can replace methionine or cystine sulfur. After feeding of $Na_2S^{35}O_4$, over 20% of the S^{35} was recovered in the chickens as taurine and what are probably taurine metabolites. Taurine accounts for a substantial part (6.7%) of the total sulfur in the chicken and it is quite widely distributed in the tissues. It is possible that free taurine has some function other than as component of bile acids(6) and therefore dietary sulfate might also be of value as a precursor of taurine.

Summary. 1. Addition of 0.5% sodium sulfate to a diet low in sulfur stimulated growth of the young chicken. 2. When $Na_2S^{35}O_4$ was fed for a period of 1 week, approximately 23% of the S^{35} ingested was incorporated into the tissues. Most of the S^{35} was recovered as sulfate-S^{35}. However, about 19% was present as taurine-S^{35}, 6.6% as unknown organic compounds and barely detectable amounts as sulfur amino acids. Two weeks after $Na_2S^{35}O_4$ feeding a larger proportion of S^{35} was present in the carcasses as taurine and unidentified organic compounds and 1.5% of the S^{35} in the chickens was accounted for as cystine and methionine. It was concluded that there is a physiological requirement for sulfate *per se* and that dietary sulfate promotes growth primarily by providing sulfur for this purpose. 3. Taurine was found to be the major sulfur constituent of the water-soluble portion of feathers.

1. Gordon, R. S., and Sizer, I. W., *Science*, 1955, v122, 1270.

2. Machlin, L. J., Jackson, J. T., Lankenau, A. H., and Pearson, P B., *Poultry Sci.*, 1954, v33, 234.

3. Machlin, L. J., Peason, P. B., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1953, v205, 213.

4. Machlin, L. J., Pearson, P. B., and Denton, C. A., *ibid.*, 1955, v212, 469.

5. Machlin, L. J., Struglia, L., and Peason, P. B., *Arch. Biochem. Biophys.*, 1955, v59, 326.

6. Bolliger, A., and Gross, R., *Australian J. Exp. Biol. Med. Sci.*, 1952, v30, 181, 399.

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Cultivation of L Strain (Earle) Mouse Cells in Bacteriological Media Combined with Horse Serum. (22709)

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Research in the development of tissue culture media has been divided into two major areas. The first is the attempt to develop completely chemically defined media(1-8). The second area of research is the attempt to substitute various processed materials for blood serum and tissue extracts currently used in many tissue culture media. These processed materials, which include yeast extract(9), peptone preparations(10-13) and serum fractions(14,15), are sometimes combined with natural body substances(16) or with chemically defined media(1,3,4,17). This paper reports the development of a tissue culture medium which incorporates commercially available bacteriological preparations as supplements to blood serum in the cultivation of Earle's L strain of mouse fibroblasts.

Materials and methods. L cells(18) grown at 37°C for 3 to 5 days were scraped from T-60 Earle flasks and washed twice with Hanks' balanced salt solution (BSS). The cells were suspended in BSS and added to the various media which were then dispensed in 1 ml aliquots to Porter flasks. The concentration of cells in the Porter flasks ranged from 100,000 to 125,000 cells per ml. The following preparations have been tested for stimulation of L cell growth: Bacto Yeast Extract, Bacto Beef Extract, Bacto Casamino Acids, Difco Proteose Peptone, Difco Proteose Peptone #2, Difco Proteose Peptone #3, Difco Neopeptone, Tryptone, Tryptose, and Heart Infusion Broth, all produced by Difco Laboratories; Phytone, and Trypticase, produced by the Baltimore Biological Laboratory and N-Z Case produced by the Sheffield Chemical Co. The preparations to be screened were prepared as a 2% solution in BSS and sterilized by filtration through fritted glass. Concentrations of 2, 1, 0.1, and 0.01% were generally tested. In addition, these concentrations in BSS were tested in combination with 10% horse serum. The standard reference medium consisted of 60% medium 199,* 20% whole egg ultrafiltrate,* and 20% horse serum. Fifty units each of penicillin and streptomycin were added to each flask. All cultures were prepared in triplicate and incubated at 37°C. Various qualitative estimates were made to evaluate the growth rate and the appearance of cells from day to day. In addition to these qualitative estimates, quantitative determinations were made by taking total cell counts on the fourth day. In other short term experiments designed to test for stimulation of growth, a daily count was made for 5 days. The total cell count was made by removing the medium and replacing it with 1 ml of 0.25% trypsin in saline. Cell removal was hastened by shaking the Porter flasks on a New Brunswick rotary-type shaker operating at 100 rpm. The cells thus removed were counted in a hemocytometer. Two total cell counts were done on each Porter flask. The values shown in the figure and in the table represent the average count of triplicate flasks of a single experiment. The results of this experiment are representative of several that were performed. Preparations that showed some enhancement of growth were combined and tested in the presence of various concentrations of horse serum.

Results. Among the products screened, yeast extract and proteose peptone #3 gave the best results. One tenth % yeast extract was found to be the optimum concentration for proliferation of L cells in the presence of horse serum. This agrees with the results of Robertson with Hela cells(9). The optimum concentration of proteose peptone #3 was found to be 0.1%. No multiplication occurred in the absence of horse serum. The growth of L cells in a medium consisting of 0.1% yeast extract and 0.1% proteose pep-

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* Obtained from Microbiological Associates, Bethesda, Md.

TABLE I. Multiplication of L Cells in Difco Bacto-Yeast Extract, Proteose Peptone #3, and Horse Serum.*

% yeast extract	% proteose peptone	% horse serum	Total cell count on 4th day × 10^5	Fold increase
.1	0	20	6.00	4.8
.1	0	10	3.44	2.8
.1	0	2.5	1.52	1.2
0	.1	20	4.49	3.6
0	.1	10	3.29	2.7
0	.1	2.5	1.35	1.1
.1	.1	20	6.63	5.3
.1	.1	10	4.99	4.0
.1	.1	5	2.29	1.8
.1	.1	2.5	1.98	1.6
.1	.1	0	.46	.4
Standard reference medium†			3.74	3.0

* Initial inoculum: 1.25 × 10^5 cells/ml.

† 60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum.

tone #3 in the presence of various concentrations of horse serum is presented in Table I. The same table shows the growth obtained when these constituents were tested separately in the presence of horse serum. The growth of cells in 0.1% yeast extract or 0.1% proteose peptone #3 in the presence of 20% horse serum exceeded growth obtained in the standard reference medium. Growth in the presence of 10% horse serum was also better than that of the standard reference medium.

Among the combinations tried, 0.1% yeast extract combined with 0.1% proteose peptone #3 in the presence of horse serum gave maximum growth and exceeded that obtained using either alone. This was true at all levels of horse serum tested. There was a 5.3 and 4.0 fold increase in the total cell count in 4 days at the 20 and 10% serum levels respectively, compared to 3.0 fold increase in the standard reference medium. Although the total cell count at serum levels below 10% did not reach that of the standard reference medium, a significant degree of cell multiplication occurred at these low serum levels. There was a 1.8 and 1.6 fold increase in the total cell count at the 5 and 2.5% levels of horse serum.

Fig. 1 shows the growth curve of L cells in these two nutrients when combined with various concentrations of horse serum. The growth rate of L cells in yeast extract and proteose peptone #3 combined with 20 and 10% horse serum exceeded the rate obtained in the standard reference medium, after 5 days incubation.

Fig. 2-4 show photographs of the appearance of L cells grown in 0.1% yeast extract and 0.1% proteose peptone #3 combined with various concentrations of horse serum. The cells are thinner and more sparse in cultures where serum has been omitted.

In preliminary experiments L cells have also been cultivated for 11 days in the standard reference medium and in the yeast extract-proteose peptone #3 combination with horse serum levels of 20, 10 and 5%. The medium was changed twice during this period and the cells were transferred from T-60 Earle flasks to Roux bottles after the seventh day of incubation. The total cell count of these cultures after 11 days incubation had increased 43.7, 47.4, 34.5, and 29.2 fold re-

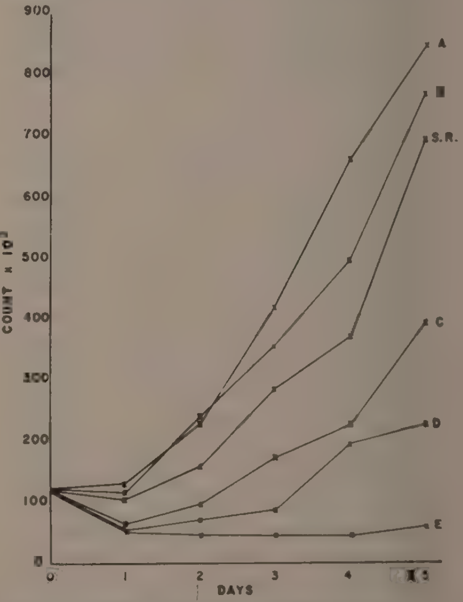

FIG. 1. Growth of L cells in yeast extract (y. e.) and proteose peptone #3 (p-p #3) combined with various concentrations of horse serum (HoS).

A. 0.1% y. e. and 0.1% p-p #3 plus 20 % HoS
B. 10 %
C. 5 %
D. 2.5%
E. 0 %

S. R. Stand. reference medium (60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum)

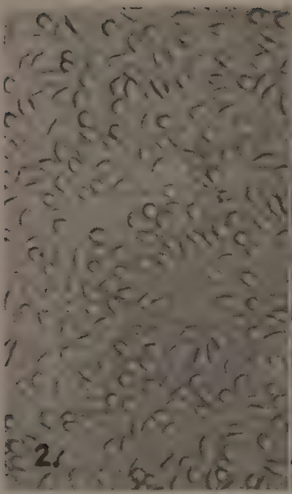

FIG. 2 (left). Earle's L strain cells from the mouse after 4 days in the standard reference medium (60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum). 100×.

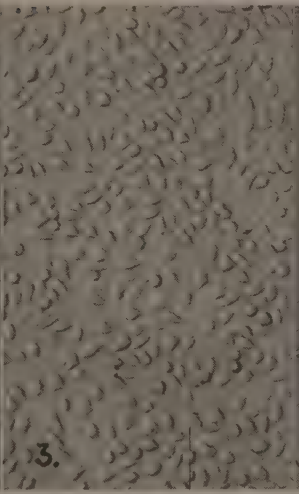

FIG. 3 (center). L cells after 4 days in 0.1% Bacto-yeast extract and 0.1% proteose peptone #3, Difco, with 20% horse serum. Growth with 10% horse serum was equally good. 100×.

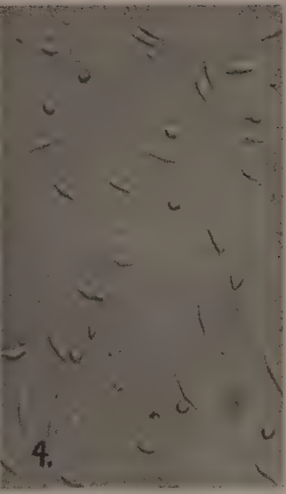

FIG. 4 (right). L cells after 4 days in 0.1% Bacto-yeast extract and 0.1% proteose peptone #3, without horse serum. 100×.

spectively. Under these conditions, the yeast extract-proteose peptone #3 combination in the presence of 20% horse serum produced slightly more growth than did the standard reference medium.

The medium reported here supported the growth of Gey's MBIII strain (mouse lymphoblasts)(19), Maben strain (a human epithelium-like cell derived from an adenocarcinoma of the lung)(20), and a strain of guinea pig lung fibroblasts that was isolated in this laboratory. Eighteen serial passages of L cells have been made in this medium containing 10% horse serum during a period of 17 weeks. The MBIII strain, Maben strain, and the guinea pig lung fibroblasts have been subcultured 13, 5, and 13 times during a 12, 6, and 18 week period respectively. The growth of these cell lines is undiminished and their appearance is normal.

The yeast extract-proteose peptone #3 medium, without horse serum, can be prepared as one solution and autoclaved.

Discussion. The present findings are consistent with the earlier reports that yeast extract and peptones support the proliferation of cells when combined with serum. The rapid multiplication of washed cells in the yeast extract-proteose peptone #3 medium reported here suggests that this medium contains growth factors that supplement blood serum and promote the growth of several lines of mammalian cells. Yeast extract is known to be an excellent source of B vitamins, and peptones are a rich source of nitrogenous compounds. The possibility that other unidentified growth factors are present in these preparations can not be ruled out.

It is significant to point out that the growth obtained in this medium, combined with horse serum, was slightly better than the growth obtained in a synthetic medium supplemented with whole egg ultrafiltrate and horse serum. The medium described here contains commercially available products that are cheap, reproducible, and convenient to handle. Their use in the large-scale cultivation of cells is therefore advantageous.

Summary. The combination of 0.1% yeast extract and 0.1% proteose peptone #3 in Hanks' balanced salt solution plus 20% horse serum stimulated the rate of growth and produced a higher total count of L cells at the end of 5 days than did the standard reference

medium (60% medium 199, 20% whole egg ultrafiltrate, and 20% horse serum). Lowering the serum content in the new medium to 10% still permitted better growth than the standard reference medium. The yeast extract-proteose peptone solution could be sterilized by autoclaving without affecting its growth-stimulating properties.

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1. Morgan, J. F., Morton, H. J., and Parker, R. C., Proc. Soc. Exp. Biol. and Med., 1950, v73, 1.

2. White, P. R., and Waymouth, C., *Ann. N. Y. Acad. Sci.*, 1954, v58, 1023.

3. Healy, G. M., Fisher, D. C., and Parker, R. C., Proc. Soc. Exp. Biol. and Med., 1955, v89, 71.

4. Eagle, H., *Science*, 1955, v122, 501.

5. ———, *J. Biol. Chem.*, 1955, v214, 839.

6. ———, *J. Exp. Med.*, 1955, v102, 37.

7. ———, Proc. Soc. Exp. Biol. and Med., 1955, v89, 362.

8. White, P. R., *J. Nat. Cancer Inst.*, 1955, v16, 769.

9. Robertson, H. E., Brunner, K. T., and Syverton, J. T., Proc. Soc. Exp. Biol. and Med., 1955, v88, 119.

10. Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, v44, 503.

11. Baker, L. E., and Carrel, A., *ibid.*, 1928, v48, 533.

12. Waymouth, C., *Anat. Rec.*, 1955, v121, 478.

13. Ginsberg, H. S., Gold, E., and Jordan, W. S., Jr., Proc. Soc. Exp. Biol. and Med., 1955, v89, 66.

14. Sanford, K. K., Waltz, H. K., Shannon, J. E., Jr., Earle, W. R., and Evans, V. J., *J. Nat. Cancer Inst.*, 1952, v13, 121.

15. Sanford, K. K., Westfall, B., Fioramonti, M., McQuilkin, W., Bryant, J. C., Peppers, E., Evans, V. J., and Earle, W. R., *ibid.*, 1955, v16, 789.

16. Fischer, A., *Acta Physio. Scandinav.*, 1941, v3, 1954.

17. Evans, V. J., Bryant, J. C., Sanford, K. K., and Earle, W. R., *Ann. N. Y. Acad. Sci.*, 1954, v58, 1012.

18. Sanford, K. K., Earle, W. R., and Likely, G. D., *J. Nat. Cancer Inst.*, 1948, v9, 229.

19. Owens, O., Von, H., Gey, M. K., and Gey, G. O., *Ann. N. Y. Acad. Sci.*, 1954, v58, 1039.

20. Frisch, A., Jentoft, V., Barger, R., and Losli, E., *Am. J. Clin. Path.*, 1955, v25, 1107.

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Evaluation of a Rotating Disc Type Reservoir-Oxygenator.* (22710)

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The historical development of various pump-oxygenators is well covered in the literature(1,2,3). Many of the early machines, utilized both experimentally and clinically, bypassed but one side of the heart, this being a simpler solution to the overall problem(4,5,6). However, Gibbon(7) and Dennis(8) have continually stressed the necessity of total cardiac bypass as a requirement for an entirely satisfactory pump-oxygenator. Lillehei and associates(9,10,11,12) hastened the reality of direct vision intracardiac surgery through the use of controlled cross-circulation, oxygenated blood reservoirs, and biological oxygenators, as temporary expedients before the final utilization of a pump-oxygenator. They also have stressed the concept of the low flow principle which has been satisfactory in their hands for shorter periods of bypass, and which possibly has simplified certain problems inherent in the development of effective pump-oxygenators.

It was the purpose of the present study to evaluate a rotating disc type reservoir-oxygenator, which has been used successfully for open intracardiac surgery experimentally and clinically.

Description of apparatus. The essential components of the present pump-oxygenator are a model TS Sigmamotor pump and a rotating disc type reservoir-oxygenator. (Fig. 1 and 2). The basic mechanism of the oxygenator is a series of 59 plastic coated stain-

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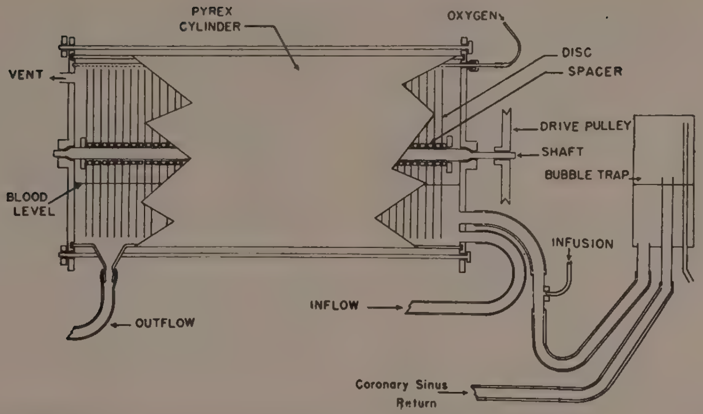
PYREX CYLINDER
OXYGEN
DISC
SPACER
VENT
DRIVE PULLEY
SHAFT
BUBBLE TRAP
BLOOD LEVEL
INFUSION
INFLOW
OUTFLOW
Coronary Sinus Return

FIG. 1. Diagram of reservoir-oxygenator with attached defoaming chamber for coronary sinus return.

less steel discs 0.15 inch (0.4 mm) thick, and 4 13/16 inches (12.2 cm) in diameter, mounted 0.18 inch (0.5 mm) apart by means of stainless steel spacers on a central shaft. Previously the stainless steel discs were coated with silicone resin; at present they are coated with teflon. The disc assembly is supported horizontally within a silicone-coated pyrex cylinder 13 inches (33 cm) long, and 5¼ inches (13.3 cm) in diameter, by gasketed end plates of stainless steel, likewise treated with silicone resin. Blood is introduced at one end of the cylinder and is removed from the bottom of the opposite end. Rotation of the discs effectively prevents channeling of the blood along the bottom of

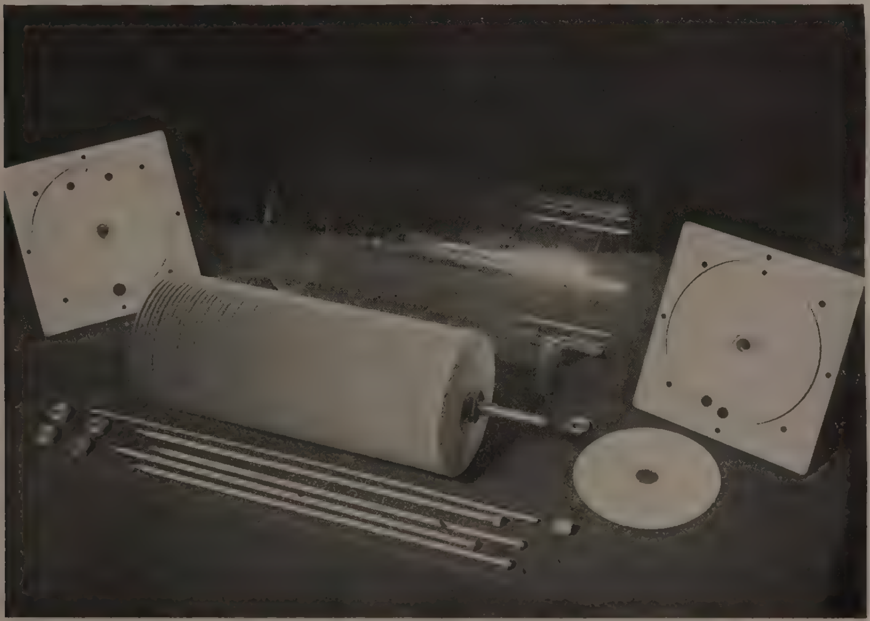

FIG. 2. Reservoir-oxygenator dissembled to show component parts.

the cylinder. The oxygenator is primed with 1400 cc of whole blood to immerse the discs to 1⅝ inches (4.1 cm), a level calculated to provide maximum exposure of blood to oxygen. When the discs rotate the blood films on the peripheral 1⅝ inches of each disc for exposure to oxygen. With the oxygenator primed with 1400 cc the static exposed area (that above the blood level) of the 59 discs is 9 square feet or 0.84 square meter. At a disc rate of 120 revolutions per minute, the rate generally employed, 1080 square feet or 110 square meters of filmed blood is exposed to oxygen per minute. To turn the discs, a pulley mounted on one end of the shaft is driven through a V-belt by a 1/80 horsepower motor controlled by a variac power rheostat. Oxygen is supplied at a rate of 5 liters per minute through a perforated stainless steel tube mounted within the cylinder above the discs. The oxygen is warmed by passing through tubing immersed in a sand-bath, heated by an electric mantle. The oxygenator itself is warmed by a wrapping of silicon rubber-covered resistance wire, the temperature of which is controlled by a variac. All electrical connections are mounted above the oxygenator, over 5 feet above the floor, to eliminate explosion hazards. Heat may be supplied selectively by increasing the current through the resistance wire and condensation within the oxygenator has been effectively prevented. Provision is made at the inlet face of the oxygenator for filling prior to use, the addition of blood to the cylinder during a run, and for sampling the venous return to the oxygenator from the patient. The output face is provided with openings to vent off the circulating oxygen, for collection of samples of oxygenated blood, and to receive a thermocouple for continuous monitoring of blood temperature. Recently a sump type suction tip has been used to collect coronary sinus return from the right atrium, utilizing a second Sigmamotor pump. Coronary sinus blood is pumped first to a defoaming chamber, from which it is returned to the input end of the oxygenating cylinder. Venous cannulations have been made with expendable nasal oxygen catheters, or with specially constructed adaptors of polyethylene, and all external tubing is tygon, 5/16 inch internal diameter, except the short sections of silicone coated rubber tubes in the pump head. To reduce turbulence and fibrin formation, all the adaptors are of high tensile, polished polyethylene, of the same internal diameter as the tubing they receive. A bubble trap has not been necessary on the outflow line since there is no bubbling present in the oxygenator, and filters in the external circuit are not used. Pump speeds are regulated through a previously calibrated speed changer. The oxygenator can easily be dissembled into its component parts for cleaning. It is sterilized in the autoclave after reassembly including the oxygen flow tube and the heating wire. The inside is then dried by flushing with oxygen or air while it is still hot, or by standing in the hot autoclave. Connecting tubes are sterilized in Zepharin for twenty-four hours and then rinsed and mounted in the pump circuit. The tubing is then rinsed in two liters of saline solution before being connected to the patient.

Method of study. Effective pump-oxygenators must maintain circulation as well as oxygenation in total cardiac bypass with a low morbidity and mortality rate. To evaluate the present pump-oxygenator, both acute and survival experiments were carried out with dogs, as well as studies utilizing cow blood. Observations carried out during the cardiac bypass were continuous mean blood pressure recordings, blood oxygen and carbon dioxide content, blood pH, plasma hemoglobin, hematocrit, and platelet and red and white blood cell counts. It was difficult to evaluate the oxygenating capacity of the oxygenator by dog experiments alone because of limitations imposed by the size of the available dogs. For this reason, experiments were done using cow blood. In these experiments, warmed desaturated cow blood was run through the oxygenator at the different flow rates to be studied. In each experiment, after 4 liters of blood had passed through the oxygenator and equilibrium had been established, samples of venous and oxygenated blood were taken and analyzed for their oxygen content. Blood samples for oxygen determination were packed in ice and the oxygen content was determined in duplicate within 24 hours by the method of Roughton and Scholander.

Results. Mortality and morbidity were necessarily high in the developmental stage of the pump-oxygenator. However, after the basic design and technics were standardized a survival series of 20 dogs was carried out in which there were 2 deaths. In 11 of the dogs, simple cardiac bypass without cardiotomy was done, with one death due to undetermined causes; and in the remaining 9 dogs a ventricular cardiotomy was done with one death due to postoperative ventricular fibrillation following the institution of a citrated blood transfusion.

Perfusion pressures measured as mean arterial blood pressures with a mercury manometer, at flow rates from 40 to 80 cc/kg/min., averaged from 50 to 80 mm Hg. To maintain such pressures it was necessary in most experiments to add varying amounts of blood to the total circulating blood volume even though there was no demonstrable extrinsic loss. This could be partly compensated for by adding small amounts of neosynephrine to the oxygenator. Although the blood pressure could be affected in some measure by increased perfusion rates, this was not constant, and initial small rises were not maintained.

TABLE I. 25 Oxygenation Experiments Using Cow Blood.

Exp. No.	Blood flow, cc/min.	Oxygen saturation vol, % Arterial	Venous	A-V diff.	cc O_2/min. added to blood
1	660	20.3	7.4	12.9	85
2	940	18.9	11.8	7.1	67
3	1060	17.6	5.0	12.5	128
4	1125	17.6	5.3	12.3	138
5	1200	14.4	1.4	13.0	156
6	1224	15.9	4.8	11.1	136
7	1240	19.1	7.4	11.7	145
8	1330	15.0	4.8	10.2	149
9	1350	17.5	11.7	5.8	78
10	1475	15.4	7.4	8.0	118
11	1500	12.3	1.9	10.4	156
12	1580	15.5	5.3	10.2	138
13	1670	15.6	5.0	10.6	177
14	1710	14.9	5.1	9.8	168
15	1920	14.7	7.6	7.1	136
16	2060	13.8	5.5	8.3	171
17	2120	15.1	11.4	3.7	98
18	2250	13.7	4.8	8.9	203
19	2500	13.2	5.1	8.1	203
20	2537	10.5	5.5	5.0	127
21	2600	10.4	3.3	7.1	182
22	2640	12.6	7.6	5.0	132
23	2860	15.8	11.8	4.0	114
24	3080	11.8	5.1	6.7	207
25	3080	16.1	11.8	4.3	132

A specific value as to the oxygenating capacity and efficiency of the oxygenator cannot be given at present. With such factors as oxygen flow rate, disc rate, and blood temperature held constant, the efficiency of the oxygenator varied not only with the blood flow rate but also with the degree of desaturation of the venous blood returning to the oxygenator. With low oxygen saturations in the venous blood the oxygenator was more efficient in that more cubic centimeters of oxygen were added to the blood per minute; but lower arterial oxygen saturations were obtained. The reverse was true with higher levels of venous oxygen saturation. This is well illustrated in experiments 8 and 9, 16 and 17, 24 and 25, in Table I. In these experiments the efficiency of the oxygenator ranged from 67 cc/min. of oxygen added to the circulating blood, to 207 cc of oxygen added per minute. The arteriovenous oxygen difference ranged from 3.7 to 13 vol. %, depending on the oxygen content of the venous blood and the blood flow rate employed. Pertinent oxygenation data from a typical dog perfusion experiment are found in Table II. There was adequate oxygenation at a flow rate of 2000 cc of blood per minute (59 cc/kg body weight per minute) with an arteriovenous oxygen difference of 6.8 vol. % and an oxygen uptake of 136 cc per minute.

Hemolysis was not excessive (Table III). The increase in serum hemoglobin over a 30 minute pumping period ranged from 10 mg% to 160 mg%. Variations in serum hemoglobin could not be related to such factors as disc speeds below 120 revolutions per minute, or pump speeds, but the degree of

TABLE II. Typical Dog Cardiac Bypass Experiment.

Wt, kg	Blood flow, cc/min.	Disc rate, r.p.m.	O_2 saturation vol, % Arterial	Venous	cc O_2/min. added to blood
34	1000	120	18.3	10.3	80
	1600	120	18.9	10.1	141
	2000*	120	18.6	11.8	136

* 59 cc/kg/min.

TABLE III. Average Increase in Serum Hemoglobin.

	Interval of bypass			
	10 min.	20 min.	30 min.	40 min.
No. of exp.	17	18	14	4
Serum hemoglobin (mg/100 cc)	29	36.4	67.7	88.5

hemolysis did increase with pumping time. At disc rates in excess of 120 revolutions per minute hemolysis was increased by the added churning, and in addition foaming occurred in the oxygenator. There was moderate destruction of platelets. In 13 experiments in which they were counted, the platelets dropped from an average of 184,770 to 96,000, or roughly to one-half of their preperfusion value over a period of 30 minutes. When protamine sulphate was given in amounts equal to one-half the calculated dose of heparin, and fresh citrated blood given postoperatively, the clotting time was restored to normal. Postoperative bleeding was not a serious complication. Leukocyte counts showed no consistent change following periods of cardiac bypass. Blood carbon dioxide content and pH studies are better controlled in the clinical cases done to date and will be discussed later.

Discussion. The oxygenator described is simple in design, easy to operate, and can be constructed to oxygenate over a wide range of blood flow rates without requiring excessive blood for priming purposes. Although it resembles the Bjork(13) apparatus in general principles, it differs in certain specific aspects of its design. More plates have been used and they are teflon coated which appears to reduce the degree of hemolysis and eliminates the tendency for fibrin formation on the disc surfaces. By increasing the number of discs and filling the cylinder to a higher level, the minute surface area of exposure to oxygen is 110 square meters compared to 44 square meters for the small oxygenator and the somewhat higher value for the large oxygenator of Bjork. The pump-oxygenator here described is considered to be the basic experimental model and it has been undergoing continual modifications which retain the basic principles of construction described. The oxygenating reservoirs are now being constructed from plexiglass and the discs are being made from teflon rather than from the stainless steel coated with teflon. In addition, a large cylinder is now available to increase the oxygenating capacity of the unit for use in adults; and a smaller cylinder is being constructed for use with infants in order to reduce the necessary priming volume. The present unit has been used in 30 clinical cases to date with no deaths specifically attributable to the pump-oxygenator. Details of this experience will be published later.

Summary. 1. A rotating disc type reservoir-oxygenator is described. 2. Its effectiveness in maintaining circulation and oxygenation is discussed. 3. It has proven satisfactory in both its laboratory and clinical application.

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1. Karlson, K. E., Dennis, C., Westover, D., Sanderson, D., *Surg.*, 1951, v29, 678.

2. Stokes, T. L., Gibbon, J. H., *S. G. & O.*, 1950, v91, 139.

3. Dennis, C., Spreng, D. S., Jr., Nelson, G. E., Karlson, K. E., Nelson, R. M., Thomas, J. V., Eder, W. P., Varco, R. L., *Ann. Surg.*, 1951, v134, 709.

4. Sirak, H. D., Ellison, E. H., Zollinger, R. M., *Surg.*, 1950, v28, 225.

5. Dodrill, F. D., Hill, E., Gerisch, R. A., Johnson, A., *J. Thorac. Surg.*, 1953, v26, 584.

6. Sewell, W. A., Glenn, W. W. L., *Surg.*, 1950, v28, 474.

7. Miller, B. J., Gibbon, J. H., Gibbon, M. D., *Ann. Surg.*, 1951, v134, 694.

8. Dennis, C. E., Discussion by; Miller, B. J., Gibbon, J. H., Jr., Greco, V. F., Smith, B. A., Cohn, C. H., Allbritton, F. F., Jr., *J. Thorac. Surg.*, 1953, v26, 631.

9. Warden, H. E., Cohen, M., Read, R. C., Lillehei, C. W., *ibid.*, 1954, v28, 331.

10. Lillehei, C. W., Cohen, M., Warden, H. E., Read, R. C., Aust, J. B., DeWall, R. A., Varco, R. L., *Ann. Surg.*, 1955, v142, 418.

11. Warden, H. E., Read, R. C., DeWall, R. A., Aust, J. B., Cohen, M., Ziegler, N. R., Varco, R. L., Lillehei, C. W., *J. Thorac. Surg.*, 1955, v30, 649.

12. Warden, H. E., Varco, R. L., Lillehei, C. W., *Am. Assn. for Thoracic Surg.*, 36th ann. meet., Miami Beach, Fla., May 7-9, 1956.

13. Bjork, V. O., *Acta Chir. Scand.*, 1948, v96, suppl. 137.

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Lack of Effect of Carbutamide on Activity of Rat Liver Glucose-6-Phosphatase. (22711)

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Carbutamide (BZ-55, or p-aminophenylsulfonyl butyl carbamide) exhibits hypoglycemic activity in normal animals and humans and in some diabetic individuals(1,2,3). One possible explanation of this physiological activity is that carbutamide interferes with the conversion of glycogen to glucose by inhibiting one of the enzyme systems involved in this conversion. The observation of Miller and Dulin(4) that liver glycogen levels are elevated after the administration of 1-butyl-3-p-tolylsulfonyl urea, an analog of carbutamide, appears to support this explanation.

Glucose-6-phosphatase, one of the enzymes involved in the conversion of glycogen to glucose, is more active in the liver of the diabetic animal(5,6), and is less active in the presence of glycogen storage disease(7). This paper describes a study on the effect of carbutamide on glucose-6-phosphatase activity of rat liver *in vitro*, and correlates these findings with the levels of carbutamide which were found in liver and plasma of animals treated with the drug.

Materials and methods. Since it has been shown by deDuve(8) and Langdon *et al.*(6) that most of the glucose-6-phosphatase activity is found in the microsomal fraction of liver homogenates, it was decided to use these sub-cellular particulate fractions as the enzyme source. Therefore active microsomal suspensions were prepared from the livers of normal rats by a method similar to that described by Langdon. These were kept frozen until used. In this condition no decrease in glucose-6-phosphatase activity was observed over a period of several weeks. The reaction mixture consisted of 0.02 M substrate, either the sodium salt of glucose-6-phosphate or β-glycerol phosphate, .02 M maleate buffer, pH 6.5, carbutamide or ammonium molybdate solution in concentrations indicated elsewhere, and the microsomal suspension derived from 40 mg of liver and containing about 0.2 mg of nitrogen. The total volume was 0.5 ml and the final pH 6.5. Carbutamide was dissolved in NaOH solution and adjusted to pH 8.0 before addition to the reaction mixture. Phosphate was determined according to Fiske and SubbaRow(9). The enzymatic activity was indicated by percent of total substrate hydrolyzed. The hydrolysis in the presence of inhibitors was compared with that of control experiments for which the activity was expressed as 100. Following the administration of various amounts of carbutamide to normal fasted rats, the concentrations in liver and plasma were estimated by the method of Bratton and Marshall(10). Blood sugar levels were determined by the method of Haslewood and Strookman(11) immediately before treatment and just before sacrifice. To gain some idea of the extent of binding of carbutamide by plasma proteins, a plasma ultrafiltration method similar to that described by Coolidge(12) was used. Fractionation of the livers of treated rats into various subcellular components was made as described by Umbreit, Burris, and Stauffer(13).

Results. The enzyme preparation was characterized by determining the effect of substrate and enzyme concentration on the extent of the reaction, by utilizing a known inhibitor of the enzyme, and by using a substrate not readily hydrolyzed by glucose-6-phosphatase. The velocity of the reaction was directly proportional to the enzyme concentration. The reaction was linear for the first 20 minutes, after which time the rate decreased quite rapidly.

For the inhibition studies, an incubation time of 15 minutes was utilized to eliminate variations caused by inactivation of the enzyme(14). The substrate concentration of 2×10^{-2} M served to minimize changes in substrate concentration during the course of the enzymatic reaction. Thus, in the inhibition studies the reaction followed zero order kin-

TABLE I. Effect of Carbutamide and Molybdate on Glucose-6-Phosphatase Activity of Rat Liver Microsomes.

Addition, moles/l	% of substrate hydrolyzed 1	 2	 3	 4	Mean	Relative activity
None	25.8	25.7	25.0	26.8	25.8	100
Carbutamide 10^{-5}	31.2	28.0	31.5		30.5	118
10^{-4}	26.6	27.4	27.2		27.1	105
10^{-3}	25.5	26.7	28.1		26.8	104
10^{-2}		22.6	22.9		22.8	88
Ammonium molybdate 10^{-4}				16.2		63
10^{-3}				2.3		9
3.2×10^{-3}				0		0
10^{-2}				0		0

TABLE II. Distribution of Carbutamide in Plasma and Liver of Rats 4 Hours after Oral Administration.

Tissue	Dosage of carbutamide, mg/kg 1000	 500	 200	 100†	 50	 25
Plasma, μg/ml	1,165	676	330	250	95	73
Plasma ultrafiltrate, μg/ml	850	460	205	93	31	21–31
Liver, μg/g	665	358	137	94	66	35
Liver water, μg/ml*	900	485	185	127	89	47
Blood sugar change, mg %	–15.5	–21.0	–17.5	–5.2	–14	+13.5

* Calculated from an assumed moisture content for liver of 74%.

† Avg of 4 rats; all others on single rat experiments.

etics. The enzyme preparation was relatively specific for glucose-6-phosphate as substrate. Under identical conditions the hydrolysis of glucose-6-phosphate was 25 times the rate of hydrolysis of sodium β-glycerol phosphate.

Addition of carbutamide in concentrations from 10^{-5} to 10^{-2} M (0.27 to 270 mg per 100 ml) had no significant effect on the activity of the enzyme (Table I). In contrast, ammonium molybdate, a known inhibitor of glucose-6-phosphatase(15), produced complete inhibition of the enzyme at a concentration of 3.2×10^{-3} M.

In Tables II and III are presented data on concentrations of carbutamide in plasma and liver after administration of the drug in doses of 25 to 1000 mg per kg orally. Plasma concentrations were higher than the concentrations in liver. When the drug was administered in doses greater than 100 mg per kg there was a fair correlation between the concentration of free carbutamide in plasma and the amounts of the drug dissolved in the water content of the liver. The latter was calculated by dividing the observed concentrations of carbutamide per g wet weight of liver by an assumed moisture content of the tissue of 74%. The concentrations observed in these *in vivo* distribution studies were all within the limits of the concentrations tested with the *in vitro* glucose-6-phosphatase assay, *i.e.* 3.3×10^{-3} to 2.4×10^{-4} M. Very little if any of the carbutamide found in the liver was bound to any particulate fraction.

TABLE III. Distribution of Carbutamide in Rat Liver Intracellular Fractions. Animals received 100 mg/kg orally; sacrificed 4 hr after treatment.

Fraction	Exp. 1 μg/g liver	Exp. 2
Whole homogenate	133.0	97.0
Nuclear	5.0	2.5
Mitochondrial	4.3	0.7
Microsomal	2.5	2.0
Supernatant	100.7	104.0

Discussion. Although it seemed reasonable to postulate that carbutamide might exert its hypoglycemic activity by inhibiting glucose-6-phosphatase, the data presented here demonstrate that carbutamide had no significant effect on rat liver microsome preparations of glucose-6-phosphatase in a range of concentrations including those found to be effective in lowering blood sugar. Also the data show that very little of the drug was bound to the liver intracellular particles. Therefore the hypoglycemic activity of carbutamide must be explained on some other basis than inhibition of glucose-6-phosphatase.

Vaughan(16) has reported a similar lack of effect of 1-butyl-3-p-tolylsulfonyl urea on the glucose-6-phosphatase activity of a rat liver homogenate. However, Hawkins, *et al.*(17) reported that oral administration of carbutamide to rats for a three-week period decreased the glucose-6-phosphatase activity of a liver homogenate prepared from these rats. No information was given regarding an immediate effect of carbutamide on the glucose-6-phosphatase activity of liver. Since the effect of carbutamide on blood sugar is observed within 2 hours, the lowered level of glucose-6-phosphatase reported by Hawkins may have no direct relationship to the hypoglycemic activity of the drug.

Summary. The concentrations of carbutamide in the liver and plasma of rats receiving a wide range of doses of the drug have been determined. When carbutamide was added to a rat liver microsome preparation in these concentrations no inhibition of glucose-6-phosphatase activity was observed.

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1. Franke, H., and Fuchs, J., *Deutsch. Med. Woch.*, 1955, v80, 1449.

2. Achelis, J. E., and Hardebeck, K., *ibid.*, 1955, v80, 1452.

3. Bertram, F., Bendfeldt, E., and Otto, H., *ibid.*, 1955, v80, 1455.

4. Miller, W. L., and Dulin, W. E., *Science*, 1956, v123, 584.

5. Ashmore, J., Hastings, A. B., and Nesbett, F. B., *Proc. Nat. Acad. Sci.*, 1954, v40, 673.

6. Langdon, R. G., and Weakley, D. R., *J. Biol. Chem.*, 1955, v214, 167.

7. Cori, G. T., and Cori, C. F., *ibid.*, 1952, v199, 661.

8. deDuve, C., Berthet, J., and Hers, H. G., *Bull. Soc. Chim. Biol.*, 1950, v32, 422.

9. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, 1925, v66, 375.

10. Bratton, A. C., and Marshall, E. K., *ibid.*, 1939, v128, 537.

11. Haslewood, G. A. D., and Strookman, T. A., *Biochem. J.*, 1939, v33, 920.

12. Coolidge, T. B., *J. Biol. Chem.*, 1940, v135, 541.

13. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, Burgess, 1949.

14. deDuve, C., Berthet, J., Hers, H. G., and Dupret, L., *Bull. Soc. Chim. Biol.*, 1949, v31, 1242.

15. Swanson, M. A., *J. Biol. Chem.*, 1950, v184, 647.

16. Vaughan, M., *Science*, 1956, v123, 885.

17. Hawkins, R. D., Ashworth, M. A., and Haist, R. E., *Can. Med. Assn. J.*, 1956, v74, 972.

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Benzyldimethylbufotenin, a Powerful Antimetabolite of Serotonin. (22712)

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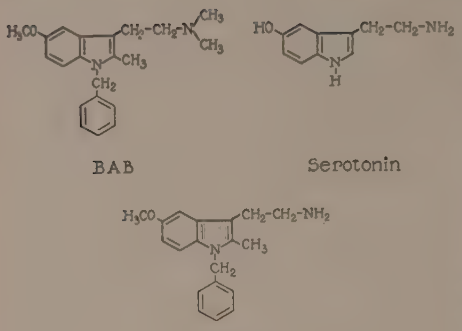
Although many antimetabolites of serotonin, capable of antagonizing specifically the actions of this hormone, have been discovered (1,2,3,4) only a few have proven to be active in whole animals. Most of them act only in isolated tissues, or exhibit only low potency in intact mammals(2,4). Even among those capable of affecting the whole animal many are only effective when administered parenterally, and fail by the oral route. Yet it is necessary to develop orally active compounds, as has been pointed out earlier(5), if useful drugs related to serotonin are to be achieved. Consequently we would like to report studies of a new antimetabolite of serotonin which is highly active in combatting the pressor action of this hormone, and one which is very effective even when fed.

The new antimetabolite is 1-benzyl-2, 5-dimethylbufotenin hydrochloride, which may be abbreviated to "benzyl analog of bufotenin" or BAB. The structural formula, as well as that of serotonin, is shown in Fig. 1. Here also is shown the benzyl analog of serotonin (BAS) which recently(4,6) has been found to be a highly active antiserotonin, capable of



BAB Serotonin

BAS

FIG. 1.

protecting dogs by the oral route from the pressor action of serotonin. BAS has also proved to be capable of lowering the blood pressure of humans suffering from hypertension(6,7), and to do so without producing marked side effects. The close relationship of the new antimetabolite to BAS is evident, hence some differences in the biological properties of BAB and BAS are noteworthy.

Preparation of BAB. BAB was synthesized as previously described(8). An unfortunate typographical error in reference(8) caused this compound to appear in the table without its methyl group at position 2. Thus, in Table II on page 4321 of the chemical paper the 12th compound should read 1-benzyl-2-methyl-5-methoxy-N, N-dimethyltryptamine hydrochloride instead of 1-benzyl-5-methoxy-N, N-dimethyltryptamine hydrochloride. A modification was introduced into the scheme of synthesis which facilitated large scale operations. This was to conduct the benzylation at the amide stage rather than at the hydrazine step. BAB in the form of its hydrochloride was soluble in water to about 40 mg per ml. In Ringer's solution or other chloride-containing aqueous solutions, it was soluble only to about 4 mg per ml. All solutions for biological testing were in Ringer's solution.

Protection of dogs intravenously against pressor action of serotonin. The tests were conducted exactly as described previously for other antiserotonins(4). Normal dogs, anesthetized with Nembutal were connected to a mercury manometer via the femoral artery, and were calibrated as to their pressor responses to graded doses of serotonin administered intravenously. Small doses of BAB (10 μg per kg) were injected intravenously into the jugular vein. The animals were then challenged again with a dose of serotonin, which, in their untreated state, had been sufficient to evoke a pressure rise of 20-40 mm of Hg. Changes in blood pressure were recorded, and if there had been no protection against the pressor effect, or if the protection was not complete, the dose of BAB was increased and the challenging repeated. Thus an estimate of the amount of BAB required to protect each dog was made. Data for 3 dogs so tested are shown in Table I. Very small doses indeed (8-12 μg per kg) were capable of causing partial protection. However complete protection was difficult to achieve, although doses of 1 mg per kg approached this goal.

In 2 additional dogs (not shown in the table) which had given abnormal responses to serotonin, the effect of BAB was less easy to assess. For example dog 35 had shown only small pressor responses to serotonin.

TABLE I. Changes in Arterial Pressure of Dogs Given BAB and Serotonin Intravenously.

Dog No.	I.V. serotonin	I.V. analog	Interval between analog & serotonin, min.	Initial pressure	Maximal pressure increase
	μg/kg			mm Hg	
7	62	0	—	102	28
	62	12	15	—	14
	62	120	15	—	12
	62	120	65	—	20
	62	1200	60	—	10
29	40	0	—	92	38
	40	8	15	—	24
	40	80	15	—	30
	40	80	75	—	38
	40	800	15	—	18
	40	800	94	—	38
34	80	0	—	116	18*†
	80	8	15	—	12
	80	1200	15	—	6

* All responses in this dog were obtained after intrav. inj. of atropine sulfate 1.2 mg/kg which was administered to obliterate the marked hypotension caused by serotonin alone.

† After each inj. of serotonin there was a very fleeting spike of pressure both before and after BAB. This response was not included in the results shown. Only sustained rises in pressure were considered.

TABLE II. Changes in Blood Pressure in Dogs Challenged with Serotonin before and after They Had Been Fed BAB.

Dog No.	Analog, mg/dog/day	I.V. serotonin challenge, μg/kg	Initial pressure	Maximal fall in pressure	Maximal rise in pressure
			mm Hg		
19	0	37	76	8	36
	10	37	78	0, 8*	26, 4*
	10	74	—	2	14
29	0	40	92	0	38
	10	40	106	20, 12*	2, 14*
32	0	71	96	0	24
	0	142	—	0, 8*	24, 30*
	10	71	70	0, 20*	22, 6†
	10	142	—	20	10

* The 2 numbers represent responses to 2 successive challenges with serotonin.

† The response to the first inj. of serotonin was followed by a rising baseline (Fig. 4, ref. 5). Subsequent challenges gave only slight rises.

These consisted of a tiny sharp spike (16 mm, 15 sec. duration) followed by a small sustained rise lasting several minutes. Larger doses of serotonin (up to 222 μg per kg) did not produce a more typical response. Administration of BAB (1.2 mg per kg) protected this dog against the sustained rise caused by serotonin, but greatly increased the height of the sharp spike (72 mm).

The protecting action of BAB appeared to be shortlived by the intravenous route. Data of Table I show that the protection had largely disappeared after about an hour. This was in contrast to the action of BAS in which case it has been found that full protection developed slowly and required about an hour after intravenous administration to become evident.

Protection of dogs by the oral route. The design of the experiments was the same as that used by Shaw and Woolley(4,5). Dogs which had been calibrated with serotonin were fed BAB once daily for 4 days. They were then challenged with serotonin again, and changes in arterial pressure were recorded and compared to those in the unprotected state. A dose of 10 mg of BAB per dog per day was found adequate to protect the animals from the pressor effects of serotonin. Data from 3 such experiments are shown in Table II.

Toxicity of BAB. The dogs which had eaten, or had been injected intravenously with BAB, showed no discernible toxic effects.

A more extensive study of toxicity was made in mice. For the acute tests, groups of 5 to 12 adult Swiss mice of the Webster strain were injected intraperitoneally with graded doses of BAB. With 100 mg/kg a flaccid paralysis of the hind legs appeared a few minutes after injection, succeeded by convulsive seizures which passed off after about an hour, followed by a period of lethargy, then complete recovery. Repetition of this dose on the following day again precipitated the signs which were again followed by recovery. After 5 days of this treatment, one of the 5 mice was unable to walk 24 hours after the last injection. Autopsy showed swollen and hemorrhagic intestines in this mouse but not in others. With 40 mg per kg only minimal signs were seen, most prominent of which were some difficulty in use of hind legs, some hyperventilation and lethargy or sleepiness which developed about half an hour after the injection. The behavioral changes caused by the large doses (100 mg/kg) could be summarized as excitement of the central nervous system, quickly followed by tranquilization.

Tests of chronic toxicity on groups of 12 adult mice were made by daily injection of 40 mg/kg intraperitoneally. Aside from slight motor difficulties with the hind legs which were noticeable for a short time after each injection, these animals showed no ill effects. They maintained their weights for the duration of the experiment (4 weeks). At this time autopsy revealed no changes which could be recognized on gross examination. The acute and chronic toxicities of BAB were therefore somewhat less than for BAS(4).

Antiserotonin action of BAB on isolated rat *uterus*. The antiserotonin action of BAB was tested in the way described for other substances(4) on the isolated uterus of estrogen-treated rats. In this system the compound prevented the contractions induced by serotonin. At 0.1 μg per ml BAB prevented the effects of 0.01 μg of serotonin per ml. Quantitative comparison of the effectiveness of BAB with that of BAS was difficult because the action was irreversible, and the potency depended on the time of exposure of the tissue to the analog before the challenging dose of serotonin was applied. These difficulties also were a prominent feature of the action of BAS, as has already been described in detail (4). The chief conclusion to be drawn from these studies was that BAB was a potent and irreversible antagonist of serotonin on the rat uterus.

Differences in action between BAB and BAS.

(a) *Taste*. BAS when applied to the tongue as solid crystals had little if any taste. BAB, on the other hand, when similarly applied had a most pronounced taste, and caused a numbing sensation which persisted and spread to adjoining areas of the tongue, and which lasted for several hours. It was reminiscent of the similar sensation caused by pyribenzamine. When one considers that BAS is a primary amine which should be attacked by amine oxidase, whereas BAB is a tertiary amine which should not be thus attacked, one may suggest that amine oxidase may play a role in the protection of nerves from the primary amine. Several of the older antimetabolites of serotonin such as the 5-aminoindoles(1) which should not be subject to amine oxidase numbed the tongue just as BAB did. The need for keeping antiserotonin out of the nervous system, for example in order to prevent psychiatric disorders, has been clearly recognized(6). A clue as to how this is to be done may be furnished by the present example which shows that, at least in the tongue, the presence of a primary aliphatic amine is necessary in an analog to keep it away from the neural structures.

(b) *Hypotension on intravenous injection*. Intravenous injection of BAS into dogs(4) or into men(7) did not result in hypotension. By contrast, doses of BAB 10 mg or greater per dog (1 mg per kg) always caused a sharp fall in arterial pressure. This was 10-40 mm of Hg (average of 6 dogs, 24 mm of Hg). The hypotension was short-lived (1-2 minutes).

(c) *Hind leg paralysis*. Mice given more than 40 mg of BAB per kg exhibited the transient paralysis of the hind legs already described. This has not been seen after treatment with BAS. This provided further evidence for an effect of BAB on nerves.

(d) In a preliminary clinical trial of BAB given orally to hypertensive patients, Drs. Robert Wilkins and William Hollander of Boston have not found it to possess the significant hypotensive effect shown by BAS at comparable dosage.

These differences in two compounds so similarly constituted, and so similar in several other biological properties tended to focus attention on the role of the primary amino-group in the designing of antiserotonins for specified pharmacological effects.

Summary. The benzyl analog of bufotenin, BAB, or 1-benzyl-2,5-dimethylbufotenin (1-benzyl-2-methyl-5-methoxy-N, N-dimethyltryptamine hydrochloride) has been found to be a highly active antagonist of serotonin. Not only was it effective on isolated tissues, but, unlike many others, was highly active as an antagonist to the pressor effects of serotonin in dogs, even when given by the oral route. Some differences in action from the corresponding primary amine (benzyl analog of serotonin) were found.

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1. Woolley, D. W., and Shaw, E., *J. Biol. Chem.*, 1953, v203, 69.

2. Erspamer, V., *Science*, 1955, v121, 369.

3. Gaddum, J. H., Hameed, K. A., Hathaway, D. E., and Stephens, F. F., *Quart. J. Exp. Physiol.*, 1955, v40, 49.

4. Shaw, E. N., and Woolley, D. W., *J. Pharm. Exp. Ther.*, 1956, v116, 164.

5. Woolley, D. W., and Shaw, E., *J. Am. Chem. Soc.*, 1952, v74, 4220.

6. ———, *Science*, 1956, v124, 34.

7. Wilkins, R. W., *New Eng. J. Med.*, 1956, v255, 115.

8. Shaw, E., *J. Am. Chem. Soc.*, 1955, v77, 4319.

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Total Serum Glycoprotein Levels in Normal New Zealand White Rabbits.*† (22713)

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Interest in the serum glycoproteins has been aroused by reports of abnormally high levels in various physiological and pathological states(1,2). Although the levels of these carbohydrate-containing proteins of the serum have been studied in many conditions and disease states, and work on establishing the chemical composition has been initiated, little is known about the physiological significance of these substances and from whence they arise(3,4).

Inasmuch as it will be necessary for laboratory animals to be used in experimental studies designed to establish the site or sites of the origin of these compounds and to determine the physiological and pathological significance of serum glycoproteins, the present investigation was undertaken to establish the normal level of serum glycoproteins in New Zealand white rabbits. In our experience, the rabbit has proven to be the most satisfactory laboratory animal for this type of study because of the similarity between its electrophoretic serum protein patterns and those of the human in both the normal and infected state.

Methods. In this series, 10 male and 9 female New Zealand white rabbits weighing from 2.3 to 2.8 kg each were used. They were individually housed in wire cages in an air conditioned room and were maintained on a diet of Purina rabbit pellets supplemented daily with carrots and tap water, *ad lib*. All animals were observed for a period of 6 weeks prior to this study in order to rule out any abnormalities. Blood samples were collected weekly on each normal healthy animal for a period varying from one to 11 weeks with the different animals. Approximately 5 cc of blood was obtained each week by lancing the ear and allowing free-flow collection in acid cleaned test tubes. With the exception of the first 3 weeks all samples were stored in the frozen state between determinations; in our experience serum specimens showed no demonstrable change for as long as 3 months. The total serum glycoproteins were determined according to a modification of Winzler's technic for protein-bound hexose determination in which 90% sulfuric acid was substituted for the 60% acid in mixing the orcinal reagent(1). Each sample was determined in duplicate on at least 2 separate occasions and the values used in the following results were derived from the average of all determinations on each sample. The mean, standard error of the mean, t, and probability values were determined by the standard statistical procedures.

Results. In 122 serum samples, the established mean and standard error for total serum glycoproteins was 84.5 ± 1.31 mg% protein-bound hexose ($p<.001$). The normal range was 56.5 to 113.5 mg% protein-bound hexose. Summaries of the results of the chemical analyses for total serum glycoproteins showing variation between individual animals and effects of weekly bleedings are presented in Tables I and II, respectively. All animals continued to gain weight throughout the course of the experiment and there were no apparent after-effects due to bleeding. No statistically significant changes occurred in the concentration of the serum glycoproteins from week to week when compared to the established mean. When compared to the first week's mean, however, there was a significant increase in the total serum glycoprotein at the sixth bleeding. No significant difference was found between sexes.

Discussion. On the whole, the variation between determinations in each animal appeared to be greater than that between the

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TABLE I. Variation between Individual Animals, Grouped According to Sex.

Male No.	No. samples	Mean ± S.E.*	Female No.	No. samples	Mean ± S.E.*
1	10	83.3 ± 3.39	24	8	80.5 ± 6.53
2	10	71.9 ± 2.81	4	10	76.0 ± 2.94
7	10	89.1 ± 5.59	6	10	84.8 ± 3.22
8	11	91.6 ± 4.68	25	9	85.6 ± 4.86
10	11	86.2 ± 2.77	12	11	90.6 ± 4.69
Avg		84.4 ± 3.42	Avg		83.5 ± 2.45

* All serum glycoprotein levels are expressed as mg % protein-bound hexose.

individual animals. The normal range varies considerably, much like blood sugar levels, but in Table I less variation is seen between the individual animals. Little change is noticed in the levels of glycoproteins in the group from week to week when compared with the average mean so that it is assumed there was no significant effect on the blood levels of glycoproteins due to the bleeding. However, when the concentrations of glycoproteins were compared to the mean of the first week a significant P value was obtained for the sixth week. We are unable to explain this observation on the basis of dehydration as total protein determinations were not done. Since the rabbits were housed in air conditioned quarters and always had ample water supply, it was not felt that dehydration was a contributing factor. On the basis of work by Weimer *et al.*(5) it appears doubtful that significant variations in total protein occur normally. They showed no significant increases, and only transient decreases in the serum proteins of guinea pigs and rats following weekly bleedings of approximately 20% of the total blood volume. Variation between animals was greatest at the third bleeding but this is unexplained and cannot be attributed to environmental changes, in that the date of the third bleeding was not the same among all rabbits. There was no significant statistical difference between the male and female groups ($P = < .001$).

TABLE II. Effects of Weekly Bleedings on Serum Glycoprotein Concentrations.

Wk No.	No. samples	Mean ± S.E.*	P†
1	9	82.9 ± 3.59	—
2	10	86.0 ± 3.06	> .2
3	10	83.7 ± 9.14	> .5
4	10	81.9 ± 4.77	> .5
5	9	85.3 ± 3.44	> .3
6	10	89.3 ± 5.00	< .05
7	8	88.6 ± 5.27	> .05
8	10	82.8 ± 3.17	> .5
9	9	82.4 ± 3.83	> .5
10	8	82.9 ± 2.03	>0
11	7	80.1 ± 3.05	> .2

* All serum glycoprotein levels are expressed as mg % protein-bound hexose.

† P values derived by comparing means of each succeeding week with mean of first week.

Summary. The normal serum levels of glycoproteins in 19 New Zealand white rabbits are reported. It was shown that there was no significant difference between the sexes and essentially little effect on total serum glycoprotein concentration when the rabbits were bled weekly in the quantity reported over an 11 week period.

1. Winzler, R. J., *Serum Glycoproteins. Methods of Biochemical Analysis II*, New York, 1955, pp 279-311.

2. Greenspan, E. M., *Arch. Int. Med.*, 1954, v93, 863.

3. Shetlar, M. R., Foster, J. V., Kelly, K. H., Shetlar, C. L., Bryon, R. S., and Everett, M. R., *Cancer Res.*, 1949, v9, 515.

4. Werner, I., *Acta Physiol. Scand.*, 1949, v19, 27.

5. Weimer, H. E., Quinn, F. A., and Redlich-Moshin, J., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 101.

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Axenic Cultivation of *Caenorhabditis briggsae* (Nematoda: Rhabditidae). V. Maturation on Synthetic Media.* (22714)

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The free-living, soil-dwelling, hermaphroditic rhabditid nematode, *C. briggsae,* can mature under axenic conditions on a variety of media containing ingredients of complex, undefined composition—for example, certain liver fractions(1-3), plasma protein fractions (4), chick embryo juice(1), and extracts of equine pancreas, thymus, or spleen(5). Some of these media have also contained a large number of added known substances(5,6), but, until recently, all have included at high levels certain of the complex, crude substances mentioned. However, we have recently recorded (7) success in rearing *C. briggsae* from larva to larva on a defined medium containing only a trace of the standard liver medium used by us in maintenance of stock axenic cultures of this organism. Now we are able to describe limited success following elimination of even this trace, such that *C. briggsae* can be reported as passing one generation on certain media of synthetic composition.

Materials and methods. Experiments were conducted as follows: 1) young adult worms were selected from stock axenic cultures maintained in standard liver medium, washed through 4 changes of 1/15 M K phosphate buffer (pH 7) in small watch glasses, and left to lay eggs for approximately 24 hours; 2) larvae hatched by the end of this period were taken up singly or in groups of 2 or 3 by micropipette and inoculated into tubes, 10 mm wide by 75 mm long, each containing 0.2-0.3 ml of liquid medium to be tested—generally only one larva was placed in a tube, 3 tubes of each medium being used; 3) tubes were plugged with cotton, sealed with 2 layers of parafilm, and incubated upright in the dark at 20°C in a constant-temperature incubator; 4) each tube was inspected daily, or every 2 to 3 days during less critical periods, placed almost horizontally on a transparent plastic square (with plugged end of tube elevated slightly) and studied under the dissecting microscope at 19.5 x magnification; 5) with each observation a record was kept, of: a) approximate size of each worm during the first generation; b) time at which the first F_1 larva or larvae were seen; c) size range of F_1 larvae during the second generation; d) time of appearance of F_2 larvae; and e) nature of the ultimate population after several weeks of incubation, determined by total or aliquot counts. We succeeded in rearing worms through one generation on 3 synthetic media, 2 being simplifications of the third. The components of the most complex medium (GM-8) are listed in Table I; its compounding is explained in Table II. The reason for using a considerable number of stock solutions in preparing GM-8 was to permit ready preparation of such simplifications of the latter as are illustrated by GM-9 and -11. Their composition is indicated in footnotes to Table I. Recent experiments leading up to, and including, those in which GM-8, -9, and -11 were tested also included testing of other defined media, the details of which will be reported later. We also tested them supplemented with various levels of standard liver medium, consisting of equal parts of liver pro-

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TABLE I. Synthetic Medium* for *Caenorhabditis briggsae*.

Components	mM	mg/l
Amino acids:		
‡L-alanine	12	1070
L-arginine (free base)	4	697
‡L-aspartic acid	10	1331
†‡L-cysteine (free base)	3	363
‡L-glutamate (monosodium)	15	2537
‡Glycine	5	375
L-histidine	4	621
L-isoleucine	8	1049
L-leucine	13	1705
L-lysinium acetate	10	2062
L-methionine	3	448
L-phenylalanine	10	1652
‡L-proline	17	1957
‡L-serine	11	1156
L-threonine	7	834
L-tryptophane	2	408
‡L-tyrosine	1	81
L-valine	11	1289
(Total)	(146)	
Other "structural" compounds:		
Choline diacid citrate	2	590
i-inositol • $2H_2O$	2	432
Nucleic acid substituents		
Adenylic acid	1	347
Guanylic "	1	363
Cytidylic "	1	323
Uridylic "	1	324
Thymine	1	126
Energy source:		
D-glucose	73	13,150
Vitamins and growth factors:		
N-acetylglucosamine		2.0
D-alanine		1.0
p-aminobenzoic acid		1.0
Biotin		.5
Cyanocobalamine		.5
Folinate (calcium)		.5
Niacin		1.0
Niacinamide		1.0
Pantetheine		.5
Pantothenate (calcium)		1.0
Pteroylglutamic acid		1.0
Pyridoxal		.5
Pyridoxamine		.5
Pyridoxine		1.0
Riboflavine-5-phosphate (sodium)		1.0
Thiamine chloride • hydrochloride		1.0
§DL-6-thioctate (sodium)		.5
‡Ascorbic acid	1	88
‖*Salts:*		
KH_2PO_4	6	817
KOH	11	617
Mg • H • citrate • $5H_2O$	2	610
$CaCl_2 \cdot 2H_2O$	1	147
K_3 • citrate	1	324
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	.1	39.2
$MnCl_2 \cdot 4H_2O$	.075	14.8
$ZnCl_2$	.05	6.8
$CuCl_2 \cdot 2H_2O$	.025	4.3
†‡H_3BO_3	.01	.62
†‡$(NH_4)_2VO_3$	.01	1.16
†‡$(NH_4)_2MoO_4 + H_2MoO_4$	.01	1.77
†‡KI	.01	1.66
Solvent (for certain vitamins):		
Triethanolamine	.057	8.5

* GM-8. † Omitted for GM-9. ‡ Omitted for GM-11. Na • citrate • $2H_2O$ at 5 mM (1471 mg per l) and citric acid at 3 mM (576 mg per l) were added to replace Na^+ and acid. § Replaced in GM-9 and -11 by thioctic acid dissolved initially at 20,000× in 5% triethanolamine (TEA).

‖ Expressed as ions:

	mM		mM
K^+	20	NH_4^+	.24
[1]Na^+	15	$SO_4^=$	.2
[2]$PO_4^\equiv$	10	Fe^{++}	.1
Mg^{++}	2	Mn^{++}	.075
Cl^-	2	Zn^{++}	.05
Ca^{++}	1	Cu^{++}	.025
		[3]Co^{++}	.00037
$MoO_4^=$, $BO_3^\equiv$, I^-, $VO_3^=$ (each)			.01

[1] As monosodium glutamate.

[2] As nucleotides + KH_2PO_4.

[3] As cyanocobalamine.

tein fraction (LPF-C)†, dissolved in 1/15M K • phosphate buffer, and an autoclaved liver infusion or extract (ALE)‡(4) prepared with the same buffer. It was added to the synthetic media at levels from 50% down to 0.032% of the level used for routine stocks, Finally we tested GM-8 supplemented separately with LPF-C and ALE at 50% and at 10% respectively. Tubes containing 100% liver medium without added ingredients were used as controls.

Results. GM-8, -9, and -11 by themselves permitted slow maturation of certain

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† Dry wt 31.88 mg/ml; N content (by micro-Kjeldahl) 2.488 mg/ml. LPF-C prepared in Aug., 1954, and stored in frozen state.

‡ Dry wt, 49.00 mg/ml; N content, 2.519 mg/ml. ALE prepared on 5-23-55 and stored in frozen state.

TABLE II. Stock Solutions* for Medium GM-8.

Stock solutions	Concentrations (relative to full-strength basal medium)
1. "Essential" amino acids	10×
2. "Non-essential" amino acids†	5×
3. D-glucose	20×
4. KH_2PO_4	100×
5. Nucleic acid bases (with KOH)	20×
6. Choline	100×
7. *i*-Inositol	100×
8. Ascorbic acid	100×
9. Vitamins: Group I	1000×
10. " II	1000×
11. " III	1000×
12. Salts‡: Group I	50×
13. " II	1000×

* All made up with triply distilled H_2O, which was also used to bring vol of medium to double strength. Latter then sterilized, usually in 10 ml amounts, by filtration through fritted glass. Stock solutions not obvious from Table I are as follows: 1. 10 amino acids essential to rat. 10. Pyridoxal • HCl, pyridoxine • 2HCl, calcium folinate, d-pantetheine. 11. Vitamins requiring initial dissolving in TEA for 20,000 × concentrated sub-stocks: pABA (5% TEA), PGA (5% TEA), niacin (1% TEA), biotin (1% TEA). 13. Salts of B, V, Mo, and I.

† Cysteine omitted; added as dry powder to final solution.

‡ $CaCl_2 \cdot 2H_2O$ omitted; added as dry powder to final solution. KOH omitted; used with nucleic acid bases.

inoculated larvae, with production of eggs by the resulting adults and hatching of F_1 larvae. On GM-11 limited growth of F_1 larvae beyond their size when newly hatched also occurred. With addition to these media of liver medium at 0.1%, maturation of the first generation (P) worms was somewhat faster, and some of the resulting F_1 larvae reached maturity and produced F_2 offspring. With liver medium at 50% the P and F_1 generations were passed rapidly, faster than in controls, but more slowly than under previously established conditions(5) in which *C. briggsae* was grown on nutrient agar slants in monoxenic association with *Escherichia coli*.

These trials, in which GM-8, -9, and -11 were tested with supplements of liver medium at 50% and 0.1% of the standard level, have been the only such experiments so far with 2 of the media—namely, GM-9 and -11. GM-8, however, was further tested with supplements at levels of 10%, 3.2%, 1%, 0.32%, and 0.032%. All worms (some 23) that were followed sufficiently long matured and produced F_1 larvae in GM-8, supplemented with liver medium at all levels down to 0.1%; but at 0.032% this was true for only one of 3 worms. At all levels except 0.032% some of the larvae of almost all progeny groups matured in turn and produced F_2 larvae. Generation I time tended to be progressively longer at progressively lower levels of supplementation with liver medium; and this was more strikingly true for Generation II time.

By contrast with liver medium, its components (LPF-C and ALE), when tested separately, proved far less active as supplements to GM-8. Starting with 3 P larva in each test, we obtained F_1 larvae with levels of both 50% [*i.e.*, the level in liver medium] and 10% for LPF-C and ALE respectively. The 50% level of LPF-C also supported the production of an F_2, but not the 10% level. By contrast, in the case of ALE the reverse was true, although only one out of the 3 tubes at 10% produced F_2 larvae and only in small numbers.

Additional quantitative data are summarized in Table III; and data from the experiments with GM-8 have been used to construct a graph expressing the activity of mixtures of it and successively smaller amounts of liver medium (Fig. 1). The lines shown were calculated by the method of least squares.

TABLE III. Typical Maturation Times in Different Media at 20°C.

Media	Maturation times in days	
	Generation I	Generation II
Synthetic media		
GM- 8	24	—
GM- 9	25	—
GM-11	17	—
Liver medium (LM)	7	5
GM-8 + .1% LM	9	19
" + 1.0% "	9	12
" + 50 % "	4.5	4
" + 50% LPF-C	6	5
" + 10% "	12.5	—
" + 50% ALE	16	—
" + 10% "	25	27
Associated with *E. coli* on nutrient agar	3.5	Not determined

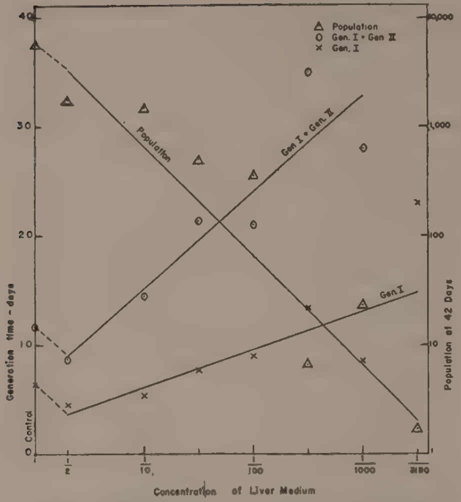

FIG. 1. Maturation time and population level as functions of concentration of liver medium in synthetic medium, GM-8.

Discussion. The foregoing results establish 3 important points: 1) it is possible to obtain slow maturation and reproduction of *C. briggsae* in certain synthetic media of known composition; 2) very small amounts of liver medium (0.032% being the lowest tested) are stimulatory to these synthetic media; but 3) liver medium must be present at much higher levels (approaching 50%) before the growth rate is equal to, or greater than, that in 100% liver medium alone.§

Even though GM-8, -9, and -11, when unsupplemented, are not adequate for indefinite cultivation of *C. briggsae*, the fact that they permit maturation and reproduction is a highly significant advance toward an adequate defined, synthetic medium. Numerous earlier attempts(5,6) with other defined media, including certain media used in tissue culture work (*e.g.*, medium 858 of Healy *et al.* (8)) had met with uniform failure; the P larvae grew very little if at all. It was not until media (GM 1-7, of which the ingredients are not given here) approaching GM-8 in composition were tried that significant growth was observed. Relatively little can be said at present as to the essentiality *vs.* nonessentiality of the various ingredients of GM-8, -9, and -11. An important point in this connection is, however, that GM-11, from which the 8 amino acids that are not required by the rat were omitted, supported better growth than GM-8, which had them.

The results with GM media, unsupplemented and supplemented with liver medium, admit of 3 interpretations: 1) the liver medium supplies fundamental growth factors that the organism cannot make, at least in adequate amounts; 2) it provides substances that have no basic metabolic role, but are needed to reverse certain toxic properties of the synthetic media; or 3) it acts in both ways.

Whatever the case may be, we feel it highly probable that a larva as originally inoculated contains a store of some highly potent substance or substances, which permit maturation of the P generation, but are insufficient to allow progress much beyond that point without the presence of liver medium. Moreover, the fact that traces of liver medium can support a relatively rapid Generation I time, yet provide for but slow growth of the F_1 larvae with a consequently much prolonged Generation II time, suggests that the original worm can efficiently utilize a trace of liver medium as a source of one or more needed substances, absent or inadequately present in the synthetic media, so long as it has an adequate store of the same or other substances carried over from the egg, but that, with the depletion of the latter store, it has difficulty in deriving its needs from the synthetic media supplemented with but traces of liver medium. Finally, the fact that the liver medium must be present at high levels to permit continued growth at a rapid rate beyond the appearance of F_1 larvae may be interpreted to mean that only high levels of liver medium can counteract the effect of depleting the original larva's store of an essential substance, or substances. It is impossible to say at the present time whether the liver medium is itself acting to supply one or more substances that the worm needs in trace amounts and that the liver medium can supply only when present at high levels, or whether some major substituent of the liver medium is needed at high concentrations, or whether both conditions obtain. In other words, it cannot as yet be said whether the liver medium is supplying one or more micronutrients, macronutrients, or both.

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§ We have found the standard liver medium to permit indefinite cultivation of *C. briggsae*, one stock of this organism now being in its fourteenth transfer after 20 months of axenic growth (July, 1956).

The results of separately supplementing the synthetic media with LPF-C and ALE provide important information. Since neither component by itself is nearly so potent as the standard liver medium, which combines the two in equal volumes, there would appear to be 2 requirements, or sets of requirements, involved—both present in each fraction, but one at relatively high levels in LPF-C and the other in ALE. The relative potency of these 2 requirements, or sets of requirements, has not yet been ascertained.

As regards *factor Rb,* a nutritional requirement for *C. briggsae* defined as protein-like and relatively heat-labile(1,7), it appears to be present in both LPF-C and ALE.

C. briggsae is the first nematode to be grown on a completely defined medium through an entire generation. Certain nematodes not belonging to the family Rhabditidae are known to be capable of axenic growth on complex media similar to our liver medium—for example, *Neoaplectana glaseri*(9); the vinegar eelworm, *Turbatrix aceti*(10) and the free-living larval stages of the dog hookworm, *Ancylostoma caninum*(11). Recently Weinstein(12) has succeeded in rearing the trichostrongyle, *Nippostrongylus muris,* of the rat to maturity on an even more complex though cell-free medium. All these species belong to the nematode order Rhabditida. It would be of great interest to test them for their capacity to grow on media similar to GM-8, with the ultimate goal (among others) of devising a rational nematocide. We ourselves have tried to grow a plant pathogen, *Ditylenchus dipsaci* (order Tylenchida), on a medium similar to GM-8, supplemented with liver medium at the 10% level, but we observed no growth of the larvae introduced into this medium.

Summary. 1. Newly hatched larvae of *Caenorhabditis briggsae* can grow to adulthood and F_1 larvae can be produced on a completely defined synthetic medium (GM-8) consisting of 18 L-amino acids, D-glucose, 4 ribonucleotides plus thymine, choline, *i*-inositol, ascorbic acid, 17 "trace" vitamins and growth factors, and a mixture of salts. 2. Media (GM-9 and -11) like GM-8 except for certain omissions (and minor additions to restore acid-base balance and salt levels) also support maturation and reproduction. 3. In the case of GM-11, omitted substances include the 8 "non-essential" amino acids. 4. The presence of a trace amount of the standard liver medium used for the indefinite axenic cultivation of *C. briggsae* is markedly stimulatory to the synthetic media, and some F_1 larvae mature and produce F_2 larvae when liver medium is present at only 0.032% of its standard strength. 5. For sustained growth, however, *C. briggsae* requires the presence of much higher levels of liver medium—between 10 and 50% of the standard level used for stock cultures. 6. Evidence is here presented that the liver medium contains 2 requirements, or sets of requirements, not present in the synthetic media, at least in adequate amounts or proportions.

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1. Dougherty, E. C., *Exp. Parasitol.,* 1951, v1, 34.
2. ———, *J. Parasitol.,* 1953, v39, 371.
3.———, *Anat. Rec.,* 1954, v120, 804.
4. Dougherty, E. C., and Keith, D. F., *J. Parasitol.,* 1953, v39, 381.
5. Dougherty, E. C., and Hansen, E. L., unpublished observations.
6. Dougherty, E. C., Raphael, J. C., Jr., and Alton, C H., *Proc. Helminthol. Soc. Wash.,* 1950, v17, 1.
7. Dougherty, E. C., and Hansen, E. L., *J. Parasitol.,* 1956, v42, pt4, sect. 2, 17.
8. Healy, G. M., Fisher, D. C., and Parker, R. C., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 71.
9. Stoll, N. R., *J. Parasitol.,* 1953, v39, 422.
10. Nicholas, W. L., *Nematologica,* in press.
11. Weinstein, P. P., *Am. J. Hyg.,* 1953, v58, 352.
12. ———, *J. Parasitol.,* 1956, v42, 215.

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Fate and Distribution of Fe^{59} Labelled Ferrocyanide in Humans and Dogs.* (22715)

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Previous studies(1,2,3) have demonstrated that unlabelled ferrocyanide ion fulfills many of the criteria required of a substance which would measure the volume of the extracellular fluid. After intravenous injection it equilibrates rapidly in a "space" approximating 20% of body weight; it does not penetrate or cannot be detected in red blood cells, gastric juice or feces; and from 94 to 98% of administered dose can be recovered in urine in 24 hours. In addition, Berliner and his associates(4) were unable to demonstrate by dialysis any binding of ferrocyanide to plasma proteins.

Unfortunately the quantities of non-radioactive ferrocyanide which must be used in "space" measurements were found by Miller and Winkler(5) to be nephrotoxic in humans. The present investigation was, therefore, undertaken to evaluate the fate and distribution in humans and dogs of small quantities of sodium ferrocyanide labelled with Fe^{59}.

Materials and methods. Studies *in vivo* were carried out in 9 human subjects and 3 nephrectomized dogs. The former included 4 male medical students, 2 patients with ascites and anasarca, one cirrhotic without ascites or edema and 2 patients with severe renal failure secondary to chronic pyelonephritis. In all experiments 30-50 mg of Fe^{59}-labelled sodium ferrocyanide (approximately 75 μc) were injected intravenously from a calibrated syringe. Measured amounts of inulin were also injected into the 3 dogs and in one subject with renal failure. Arterial samples of blood from the dogs and venous samples from the humans were collected at intervals varying from 10 minutes to one hour. Time required to reach equilibrium and the rate of disappearance of radioactivity from the blood were determined and the apparent volumes of distribution at equilibration were calculated by extrapolating back to zero time in the usual way[†](1). An aliquot of the injected solution was counted simultaneously with all biologic samples, to correct for physical decay. In most studies urines were collected for 24 to 48 hours to determine the recovered radioactivity, and in addition, in 5 experiments hourly urine collections were made to determine simultaneous renal clearances of creatinine and radioactive ferrocyanide. Radioactivity in the 48-hour stool collections of 3 subjects and in the gastric juice and saliva of one subject was also measured. The distribution of radioactive ferrocyanide between plasma and red blood cells was determined *in vivo* and *in vitro* utilizing technics previously described(1). The diffusibility of radioactive and non-radioactive ferrocyanide from aqueous solutions and plasma *in vitro* was evaluated by two technics: 1) a simple dialysis procedure in which the sample to be dialyzed was placed in a bag of Visking membrane and dialyzed against a solution of isotonic saline for 24 hours with continual shaking, and 2) the anaerobic ultrafiltration technic of Lavietes(6). The *in vivo* and *in vitro* binding of radioactive ferrocyanide by specific plasma proteins was determined by electrophoresis of samples of serum on paper at pH 8.6 and 7.4 in acetate buffer (.04M) on a Spinco apparatus at 0.6-0.7 MV. Duplicate strips of each sample of serum were run simultaneously. One was stained with bromophenol blue dye to locate the distribution of

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[†] The Donnan factor for $\mathrm{Fe\ (CN)_6^{\equiv}}$ is 1.22, *i.e.* the concentration of $\mathrm{Fe\ (CN)_6^{\equiv}}$ in interstitial fluid is 1.22 × (concentration in plasma water). Since approximately 25% of extracellular water is contained in plasma at equilibrium, the "Average" concentration of $\mathrm{Fe\ (CN)_6^{\equiv}}$ in extracellular fluid (plasma water + interstitial water) equals $\frac{(3 \times 1.22) + (1 \times 1.00)}{4} = 1.16 \times$ (concentration in plasma water).

the proteins, the other was cut transversely into 2 cm. pieces, and both were counted in a well-type scintillation counter. A solution of $Fe^{59}\ (CN)_6^{\equiv}$ in isotonic saline was simultaneously electophoresed to determine the distribution on the paper of non-protein-bound ferrocyanide ion. Total proteins were precipitated from sera using 10% trichloracetic acid. All samples were counted in duplicate for one to 5 minutes depending on the level of radioactivity. The minimal counts exceeded those of the background by at least four times. The average background with the apparatus used was approximately 115 counts per minute. Plasma and transudate proteins were determined by the biuret technic(7); inulin, endogenous creatinine, and non-radioactive ferrocyanide by methods previously described(1).

Results. Urinary recovery and renal clearance of radioactive ferrocyanide: In the 4 normal subjects an average of 80% (68-87%) of administered radioactivity was recovered in 24 to 48 hours. This contrasted sharply with the 94 to 100% of non-radioactive $Fe\ (CN)_6^{\equiv}$ recovered in 24 hours when administered to normal dogs(1) and infants (3). In Table I are listed the simultaneous renal clearances of creatinine and $Fe^{59}\ (CN)_6^{\equiv}$ in 4 normal subjects and one patient (M.S.) with severe chronic renal disease. The ferrocyanide clearance ranged from 20 to 53% of the concomitant creatinine clearance. The highest percentage was in the subject with renal disease.

Distribution between plasma and red cells.

TABLE I.* Simultaneous Clearances of Creatinine and $Fe^{59}\ (CN)_6^{\equiv}$.

Subject	Creatinine clearance, cc/min.	Ferrocyanide clearance, cc/min.
C.K.	118	41
R.S.	130	48
M.W.	122	34
M.G.	125	25
M.S.	5.6	3.0

* Each value represents the mean of 4-5 consecutive periods of urine collection. Creatinine and $Fe\ (CN)^{\equiv}$ clearances were corrected for serum water; $Fe\ (CN)_6^{\equiv}$ clearance has also been corrected for a Donnan factor of 1.22.

TABLE II. Difference between Measured and Expected Concentrations* of $Fe^{59}\ (CN)_6^{\equiv}$ in Whole Blood.

Samples	% difference (expected-measured)
7 human *in vivo*	+3.6 ± 3.9†
3 " *in vitro*	+ .3 ± .7
4 dogs *in vivo*	+1.0 ± 2.4

* "Expected concentration" in cpm/cc whole blood = (plasma concentration) (1-Hct), assuming no penetration of red blood cells by radioactive ferrocyanide in the plasma.

† Stand. dev.

Absence in gastrointestinal secretions. Table II: As had been previously noted with non-radioactive ferrocyanide(1), there was no significant penetration of the radioactive ion into the red cell. This held true whether the tracer was added *in vitro* to whole blood at 4° and 37°, or injected intravenously into humans and dogs. In addition, when red cells, from whole blood which had been labelled with ferrocyanide, were washed with cold isotonic saline and then resuspended in saline they contained no appreciable radioactivity. No significant radioactivity was detected in pooled feces, saliva, or gastric juice of humans or dogs.

Disappearance curves and apparent volume

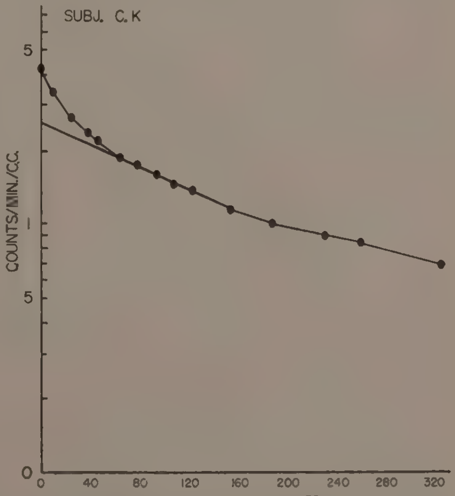

FIG. 1. Semilogarithmic disappearance of Fe^{59} labelled ferrocyanide from the plasma of a normal subject.

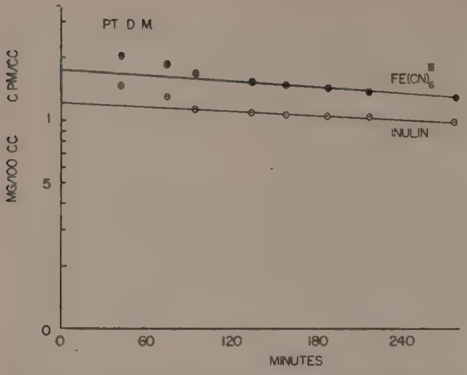

FIG. 2. Simultaneous semilogarithmic disappearance of Fe^{59} labelled ferrocyanide and inulin from the plasma of a patient with severe chronic renal disease.

of distribution in humans and nephrectomized dogs. Fig. 1 illustrates the typical semilogarithmic curve of disappearance seen in a normal subject after apparent equilibration. The disappearance curve was linear between 60 and 160 minutes. Subsequent points fell slightly above the extrapolation of the 60-160 minute line. This lack of linearity after 160 minutes was similar to that seen in normal dogs after injection of non-radioactive ferrocyanide(1). As would be expected; the rate of disappearance of the tracer was much slower in a subject with severe chronic renal disease (Fig. 2). In contrast to normal subjects, neither patient with renal disease demonstrated a changing slope in the late portion of the curve. The similarity between the rate of disappearance of inulin and ferrocyanide is apparent (Fig. 2).

TABLE III. Apparent Volumes of Distribution of Ferrocyanide and Inulin.

Subject	$Fe^{59}(CN)_6^{\equiv}$, % body wt	Inulin, % body wt
C.K.	14.0	
M.G.	15.0	
M.W.	12.7	
R.S.	10.4	
M.S.*	17.0	
D.M.*	20.0	22.5
Dogs		
1	17.1	15.1
2	16.4	19.8
3	10.6	14.0

* Subjects with chronic renal disease.

In Table III are listed the apparent volumes of distribution of radioactive ferrocyanide as well as the volume of distribution of inulin in the cases where this was determined. Only in the two subjects with chronic renal disease did the ferrocyanide "space" exceed 15% of the body weight. Both of these patients were undernourished and had slight ankle edema. Two of the normal subjects (M.W. and R.S.) had ferrocyanide "spaces" which were obviously abnormally small. In 2 of the dog experiments and in one of the patients with renal failure the inulin space actually exceeded the simultaneously measured radioactive ferrocyanide "space." These findings are in marked contrast to those of studies utilizing large (1-1.5 g) doses of non-radioactive ferrocyanide in which the ferrocyanide space always exceeded the inulin space and averaged 20% of body weight(1,2). One explanation of this discrepancy might be partial binding of the tracer dose of ferrocyanide to plasma proteins, resulting in a higher apparent concentration of ferrocyanide in plasma water than in other extracellular fluids.

To investigate this possibility *in vivo*, $Fe^{59}(CN)_6^{\equiv}$ was injected intravenously into 2 subjects with anasarca. Samples of plasma, ascitic, pleural and peripheral edema fluid were simultaneously removed at intervals of two to 36 hours after injection. The results have been expressed in Table IV as the ratio of cpm/cc of transudate water to cpm/cc of plasma water.

At equilibrium, correcting for the Donnan factor, the ratio should approximate 1.22. At no time did the ratio exceed 1.00. This held true even when the plasma concentration had fallen below a level previously attained in the transudate. This strongly suggested *in vivo* binding of radioactive ferrocyanide to plasma proteins.

Direct measurement of binding to plasma proteins: Dialysis and ultrafiltration technics were found unsatisfactory for evaluating the problem of protein binding. When samples of plasma containing radioactive ferrocyanide were dialyzed against isotonic saline for as long as 48 hours very little radioactivity could be detected in the saline. Similarly, when the plasma was ultrafiltered under a positive pressure of 35 cm of mercury, less than 30% of the expected radioactivity crossed the membrane. These observations did not necessarily indicate binding of ferrocyanide to protein. Surprisingly enough, aqueous solutions of radioactive ferrocyanide similarly did not freely diffuse or filter across a Visking membrane. This could not be corrected by dissolving the tracer in isotonic saline rather than water or by soaking the Visking membrane for 24 hours prior to use in non-radioactive ferrocyanide. Large amounts of residual radioactivity could be detected on the membrane after the latter had been washed for 2 to 3 minutes in running water.

As a result of these findings, electrophoretic and protein-precipitation technics were utilized. When aqueous solutions (water or saline) of radioactive ferrocyanide were electrophoresed at pH 8.6 and 7.4 no significant quantity of radioactivity was detected on the paper with the exception of the end dipping into the anodal bath. In contrast, when serum containing radioactive ferrocyanide was electrophoresed at pH 8.6 approximately 5% of the applied radioactivity was found to migrate with the albumin fraction, and at pH 7.4, approximately 12-18% of the activity migrated with this fraction. No appreciable binding to the globulin fractions was noted.

Little attempt was made in the present investigation to study quantitatively the nature of this binding. However, when $Fe^{59}\ (CN)_6^{\equiv}$ was added to serum samples containing increasing amounts of non-radioactive ferrocyanide the fraction bound to albumin remained approximately 5% (pH 8.6) at final ferrocyanide concentrations of 1, 2, 5 and 10 mg per 100 cc. At 50 mg per 100 cc the fraction bound fell to 1%. These findings suggest that increasing absolute amounts of ferrocyanide ion are bound up to about 10 mg per 100 cc and that above this level probably little additional binding occurs.

Further evidence of protein binding was obtained by precipitating the plasma proteins with 10% trichloracetic acid. Approximately 25% of the total radioactivity in 1 cc sample of serum remained bound to the precipitated proteins after the latter had been washed 3 times in trichloracetic acid.

Discussion. In the present study the assumption was made that the radioactivity recovered in serum, urine and transudates represented the injected Fe^{59} as ferrocyanide and that the latter was not appreciably altered in the body. The quantitative recovery of nonradioactive ferrocyanide in previous studies (1,3) suggest that this assumption is a reasonable one.

The rate of disappearance of the labelled ferrocyanide from its initial volume of distribution can be directly determined from Fig. 1. In this normal human subject the half-time value (T ½) was approximately 135 minutes. In contrast, the T½ for unlabelled ferrocyanide ion in normal dogs is approximately 40-50 minutes(1). In spite of the slower rate of disappearance in the human experiments linearity was not maintained after 160 minutes. This observation would itself constitute a strong objection to the use of the single injection technic as a method of measuring extracellular fluid volume with this tracer. One explanation for this phenomenon is that the very rapid fall in the level of ferrocyanide in the plasma causes a changing disequilibrium between the plasma and the other extracellular compartments(1). At higher concentrations, rapid renal excretion results in an increasing gradient between these compartments while at low levels the rate of transfer of $Fe\ (CN)_6^{\equiv}$ from interstitial fluid to plasma may exceed the rate of excretion by the kidney. When renal excretion of ferrocyanide is impaired, as in subjects with renal disease (Fig. 2), the conditions of equilibrium are approached more closely, and a slow, linear disappearance rate is maintained.

The most important reason for the more rapid rate of disappeaarnce observed in dogs as compared to humans(1) is the higher renal clearance of ferrocyanide ion in this species. Numerous studies in the dog(4,8) have demonstrated that the clearance of ferrocyanide is equal to the glomerular filtration

TABLE IV. Ratio of Concentration of $Fe^{59}(CN)_6^{\equiv}$ in Transudate Water/Concentration in Plasma Water.

Subject	Fluid	Time (hr)	Ratio*
M.P.	Ascitic	2	.22
		8	.63
		24	.90
		36	.90
P.G.	Edema	2	.42
		24	.85
		36	.90
P.G.	Pleural	2	.26
		24	.65
		36	.87

* Expected ratio at equilibration = 1.22.

rate. The results of the present investigation confirm the observations of Miller and Winkler(5) that the renal clearance of ferrocyanide in humans is considerably lower than the concomitant clearance of endogenous creatinine. The absolute values listed in Table I must be accepted with reservation because of the probability of significant binding of the radioactive ferrocyanide ion by the plasma proteins.

In spite of the fact that radioactive ferrocyanide did not penetrate red blood cells or enter gastrointestinal secretions, the demonstration of appreciable *in vitro* binding to plasma albumin makes it an unsuitable tracer substance for the measurement of extracellular fluid. The abnormally low volumes of distribution (Table III) and the failure of equilibration with either pleural, ascitic or edema fluid (Table IV) would support this contention. The reported similarity between the initial volume of distribution of ferrocyanide and inulin "space" or "extracellular fluid space" when large amounts of unlabelled ferrocyanide are injected is probably explained by saturation of the ferrocyanide binding capacity of plasma protein at relatively low plasma concentrations (10 mg %) of ferrocyanide. When the absolute amount of ferrocyanide injected approaches tracer quantities, however, the fraction bound to protein becomes appreciable and introduces a significant error.

The demonstration by electrophoresis that at pH 7.4, 12-18% of the added radioactivity migrated with the albumin fraction must be considered at best a rough approximation of the magnitude of the binding which might have occurred *in vivo*. The inability to use dialysis or ultrafiltration technics and the relatively low level of radioactivity in the blood at equilibration prevented adequate study of protein binding in the *in vivo* experiments.

The character of the protein-ferrocyanide bond is not clear. It does not represent combination of iron with the iron-binding-protein of the plasma, for the latter is a β globulin (9). It may represent an example of the tendency of plasma proteins to bind a large number of inorganic anions. Human serum albumin has been shown to combine according to the law of mass action with several inorganic anions(9). This binding is to the positive charges in the protein molecule and decreases as the pH of the solution increases. A similar effect of pH was evident in the present study, since less ferrocyanide was bound at pH 8.6 (5%) than at pH 7.4 (12-18%).

Summary. 1) The fate and distribution of Fe^{59} tagged ferrocyanide were evaluated in humans and dogs. 2) The tracer could not be detected in red blood cells, gastric juice, saliva or feces. 3) The apparent volume of distribution of the tagged ferrocyanide was frequently smaller than the assumed extracellular space, and in 3 instances was less than the simultaneously measured inulin space. 4) *In vitro* binding to plasma albumin was demonstrated by electrophoretic and protein precipitation technics. 5) Because of its binding to plasma protein and rapid excretion by the kidneys, radioactive ferrocyanide is considered unsuitable as a measure of extracellular fluid.

The authors wish to thank Mrs. Eva Taborsky for her invaluable technical assistance and Dr. Willoughby Lathem for his help with the electrophoretic studies.

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1. Kleeman, C. R., Epstein, F. H., Rubini, M. E., and Lamdin, E., *Am. J. Physiol.*, 1955, v182, 548.

2. Epstein, F. H., Kleeman, C. R., Rubini, M. E., and Lamdin, E., *ibid.*, 1955, v182, 553.

3. Calcagno, P. L., Husson, G. S., and Rubini, M. E., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 309.

4. Berliner, R. W., Kennedy, T. J., and Hilton, J. G., *Am. J. Physiol.*, 1950, v160, 325.
5. Miller, B. W., and Winkler, A., *J. Clin. Invest.*, 1936, v15, 489.
6. Lavietes, P. H., *J. Biol. Chem.*, 1937, v120, 267.
7. Gornall, A. G., Bardawill, C. J., and David, M. M., *ibid.*, 1949, v177, 751.
8. Van Slyke, D. D., Hiller, A., and Miller, B. F., *Am. J. Physiol.*, 1935, v113, 611.
9. Klatz, I. M., in *The Proteins*, Vol. I. Part B. 1953, Academic Press, Inc., New York.

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Modification of Viral Synthesis in Tissue Culture by Substituting Pyruvate For Glucose in the Medium.* (22716)

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A previous publication(1) has shown that maximal propagation of the PR-8 strain of influenza virus by *in vitro* cultures of chorioallantoic membrane was dependent on readily available sources of energy. Recent studies revealed that penetration of the virus and utilization of cellular energy for viral synthesis were dependent also on adequate concentration of K^+, and that pyruvate provided a more sensitive system than glucose for studying relation of potassium deficiency to virus synthesis(2). It has now been observed that, while glucose as sole carbon source for cells permits them to propagate both the parent WS and the derived neurotropic NWS strains of influenza virus, the substitution of pyruvate for glucose as carbon source permits propagation of only the parent strain WS. It appears the differences between these two strains depends not solely on host cells chosen, but also upon subtle differences in relation between viral synthesis and sources of energy for the cells.

Materials and methods. Virus. Influenza A viruses, strains WS(3) and NWS(4), were maintained by propagation in the allantoic sac of 10- to 11-day-old chicken embryos. All viral inocula were from allantoic fluid stored in hermetically sealed vials in a dry ice chest. Dilutions were made in phosphate buffer pH 7.3 to 7.4. Egg infectivity titrations (EID_{50}) and reciprocal hemagglutinin titrations (HA) were conducted by usual methods(5,6). Tissue cultures were inoculated with 10^3 to 10^4 EID_{50}. All data represent average values of 3 experiments. The 112th allantoic sac passage of strain WS was received through the courtesy of Dr. R. R. Wagner. The 114th allantoic sac passage of strain WS was used in all experiments. Strain WS showed no neurotropic properties on intracerebral inoculation of mice. Lyophilized 8th mouse brain passage of strain NWS was kindly provided by Dr. R. W. Schlesinger. The 3rd allantoic sac passage of strain NWS was used in all experiments. In 7 subsequent intracerebral passages in mice strain NWS produced a uniformly fatal disease. *Tissue cultures and substrate.* Minced and washed chorioallantoic membranes from 10- to 12-day-old chicken embryos were used in all experiments, as previously described(2). Balanced salt solution (BSS)(7) without glucose and $NaHCO_3$ was used as the basal solution. Reagent grade† carbohydrates, pyruvate, etc. were used. The final medium was adjusted to pH 7.6 with sterile NaOH before addition of tissue and virus.

Results. Table I summarizes data showing that chorioallantoic membrane tissue cultures (CamTC) maintained in 0.1% glucose supported multiplication of influenza virus

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† Nutritional Biochemical Corp., General Biochemicals, Inc., Schwarz, and California Foundation for Biochemical Research.

TABLE I. Multiplication of Strains WS and NWS in Tissue Cultures Provided with Pyruvate and with Glucose.

BSS plus:															
Pyruvate	0			.1%			0			0			.1 %		
Glucose	0			0			.1%			.02%			.02%		
	HA			HA			HA			HA			HA		
Virus (hr)	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
WS	<2	4	8	4	16	40	8	32	64	2	8	16	16	128	256
NWS	<2	<2	<2	<2	<2	<2	<2	32	64	<2	<2	±2	<2	128	128

BSS = Balanced salt solution without carbon source. HA = Reciprocal of avg viral hemagglutinin titers of 3 exp.

strains WS and NWS as measured by increases in HA, whereas in 0.1% pyruvate only strain WS multiplied. Cells in BSS without pyruvate or glucose supported multiplication of strain WS comparable with data obtained with strain PR-8 under similar conditions(1). CamTC in 0.02% glucose did not yield a significant increase in HA of strain NWS, however combination of 0.02% glucose and 0.1% pyruvate gave the highest HA observed for both viruses. In other experiments up to 5% pyruvate did not result in multiplication of strain NWS. In the presence of 0.1% pyruvate EID_{50} of tissue plus supernatant fluid determined at 12, 24, and 36 hours showed less than a 10-fold increase in infectivity of strain NWS, whereas increases of 1000-fold and greater were found with strain WS.

That the failure of CamTC to synthesize NWS virus, in presence of pyruvate, was not due to failure of virus to infect the cells, was shown in the following experiments similar to those previously reported in studies on potassium and infection with strain PR-8(2). Strain NWS at a titer of 10^3 EID_{50} was incubated with chorioallantoic membrane in the pyruvate medium for 2 hours and then anti-NWS rabbit serum was added. When the tissue was washed with physiological saline to remove the immune serum and fluids were replaced with 0.1% glucose medium, HA of 16 were detected after 72 hours. This result indicated that in the pyruvate medium strain NWS incubated with tissue became attached to cells (or penetrated cells) and was thus made insusceptible to neutralization by antibody.

The synergism observed with the combination of 0.02% glucose and 0.1% pyruvate suggests that glucose supplies factors essential for synthesis of strain NWS in CamTC. Compounds comparable to glycolytic products, Krebs cycle intermediates, high energy phosphates, coenzymes, etc. were tested for their influence on multiplication of strain NWS in CamTC. The data in Table II show that when CamTC was maintained in the presence of glucose, xylose, or glycerol, an increase in HA was observed. Ribose, dihydroxyacetone, glycerophosphate, adenosine-5

TABLE II. Effect of Various Substrates on Multiplication of Strain NWS in Tissue Cultures.

BSS +	.1%	.02% + .1% pyruvate
Substrate	HA	HA
None	<2	
Na pyruvate	<2	
Glucose	32	128
D (+) Xylose	8	8
D (−) Ribose	<2	16
D (−) Arabinose	<2	<2
Dihydroxyacetone	<2	32
Glycerol	32	32
Na glycerophosphate	<2	8
NH_4 lactate	<2	<2
Na acetate	<2	<2
Adenine sulfate	<2	<2
Adenosine	<2	<2
″ -5 phosphate	<2	32
″ diphosphate	<2	32
″ triphosphate	<2	32
Diphosphopyridine nucleotide	<2	64
Triphosphopyridine nucleotide	<2	4
Glutamine	<2	<2
Ca gluconate	<2	<2
Fumaric acid	<2	<2
Cis-aconitic acid	<2	<2
Isocitric acid	<2	<2
Malic acid	<2	<2
Succinic acid	<2	<2
Keto-glutaric acid	<2	<2
Oxalic acid	<2	<2

BSS = Balanced salt solution without carbon source.

HA = Reciprocal of avg viral hemagglutinin titers of 3 exp. after 48 hr.

phosphate, adenosine diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, or triphosphopyridine nucleotide in combination with pyruvate resulted in an increase in HA, though they were without effect when tested individually as the sole carbon source. Results with arabinose, lactate, acetate, adenine, adenosine, glutamine, gluconate, fumaric acid, cis-aconitic acid, isocitric acid, malic acid, succinic acid, ketoglutaric acid, or oxalic acid were negative when tested as a sole carbon source and in combination with pyruvate.

Discussion. The data in Tables I and II suggest that propagation of strain NWS in cells of the chorioallantoic membrane may require nucleotides or carbon sources not supplied by cells in the pyruvate medium, which nevertheless support growth of the parent strain. The difference in requirements between strains NWS and WS may or may not be a variation associated with neurotropism. This can only be determined after the growth of other influenza strains on CamTC in pyruvate and glucose has been studied. It is believed that with CamTC in a pyruvate medium adsorption and penetration of strain NWS is possible but viral synthesis is markedly reduced. Diphosphopyridine nucleotide (coenzyme I) with pyruvate in CamTC had a greater effect on virus multiplication than triphosphopyridine nucleotide (coenzyme II) which might suggest greater specificity for coenzyme I. Possibly a coenzyme I linked reaction is indicated.

Summary. Chorioallantoic membrane tissue cultures (CamTC) in balanced salt solution (BSS) with pyruvate as the sole carbon source supported synthesis of influenza virus (strain WS) but not its neurotropic variant (strain NWS). Various other substrates were studied alone and together with pyruvate for their effect on synthesis of strain NWS in chorioallantoic membrane. Cells maintained in BSS containing glucose, xylose, or glycerol as the sole carbon source supported viral synthesis, while cells maintained in ribose, dihydroxyacetone, glycerophosphate, adenosine-5 phosphate, adenosine diphosphate, adenosine triphosphate, or diphosphopyridine nucleotide supported viral synthesis only in combination with pyruvate. It is suggested that synthesis of strain NWS in CamTC requires nutritional factors which are not supplied by cells maintained in a medium with pyruvate as the sole carbon source.

1. Daniels, J. B., Eaton, M. D., and Perry, M. E., *J. Immunol.*, 1952, v69, 321.
2. Levine, A. S., Bond, P. H., Scala, A. R., and Eaton, M. D., *ibid.*, 1956, v76, 386.
3. Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Lancet,* 1933, v2, 66.
4. Stuart-Harris, C. H., *ibid.,* 1939, v1, 497.
5. Reed, L. H., and Muench, H., *Am. J. Hyg.,* 1938, v27, 493.
6. Salk, J. E., *J. Immunol.,* 1944, v49, 87.
7. Hanks, J. H., and Wallace, R. E., Proc. Soc Exp. Biol. and Med., 1949, v71, 196.

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Role of Mammary Glands on Weight of Suckling Rats. (22717)

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In nutritional studies there is a great need for employing rats showing uniformity in weight. In the weanling rats reared in these Laboratories it has not been possible to obtain appreciable uniformity in weights. The individual variation was considerable although the average weaning weight of animals born during different periods remained fairly constant.* It is now known(1) that the weaning weight of rat is influenced by the following factors: (a) the number of young the mother suckles, (b) the position of the litter in litter series, (c) the nature of the diet. While studying these aspects, it was noticed that

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* Unpublished data.

TABLE I. Relationship between "Functional" Mammary Glands and 14th Day Weight of Offsprings.

No. of functional mammary glands	8	9	10	11	12
No. of females	30	46	78	37	30
14th day wt of 6 young (g)	124.3	128.4	132.3	140.0	139.1
S.E.	2.69	2.93	2.10	2.88	2.99
Avg birth wt of 6 young (g)	29.91	30.11	30.16	30.30	30.37
S.E.	0.212	0.211	0.398	0.389	0.363

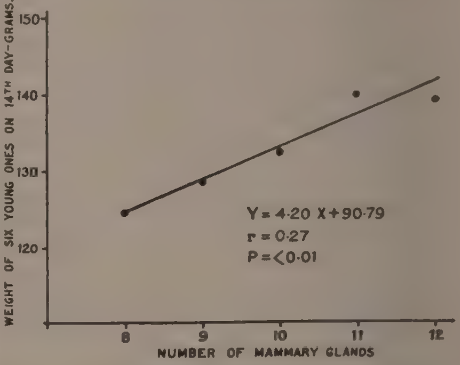

FIG. 1. "Functional" mammary glands and body wt of young.

there was some relationship between the number of well developed mammary glands of the lactating female and the weaning weight of the young. The present communication attempts to show this relationship.

Material and method. Albino rat mothers from the stock colony between the ages of 5 and 12 months, each nursing 6 young ones, were randomly selected. On the 14th day, a careful examination of their mammary glands was carried out, and the number of teats being suckled by the young ones noted. Normally, a female rat possesses 6 pairs of teats, 3 in the thoracic and 3 in the abdominal region. The teats which contain milk project prominently due to frequent sucking by the young. It is possible to exude milk from these "active" or "functional" nipples when they are gently squeezed, while those not prominent appear to be completely devoid of milk. The milk containing glands are easily distinguishable from those not containing milk as the former besides projecting prominently are marked by a circular hairless area immediately around it. On the 14th day when the number of active mammary glands was counted the body weight of young also was recorded. The reason for weighing the young on the 14th day instead of on the weaning day, *viz.*, 21st day, is that up to this day the young are known to be entirely dependent upon the mother's milk(2). Any variation in their weights at this time could be taken as a criterion of the lactating capacity of the mother rat.

Results. Relation between number of mammary glands and weight of young rats: Table I shows that the total weight of 6 young on the 14th day increases correspondingly with the increase in the number of "active" mammary glands. This is found to be statistically significant. The trend can be best described by a linear regression $Y = 4.20\ x + 90.79$, as depicted in Fig. 1. It is quite likely that the birthweight and the position of the litter series may to some extent determine the 14th day weights of young although the experimental evidence does not substantiate this, as is evident from Table I. This is what should be expected, since while the 14th day weight is dependent upon the available milk from the mother and probably the number of "functional" mammary glands, the average birth-weight is dependent upon the weight, general condition of the mother and the position of the litter series.

Number of mammary glands and position of the litter series: It has been observed in these Laboratories that the weaning weight of rats shows an increase with the order of birth. In order to investigate whether the above fact could be attributed to a corresponding increase in the number of functional glands, the data collected on the same 221 female rats were regrouped according to the order of birth, and summarized in Table II. It is clear from Table II that the average 14th day weight of the young born in the first lit-

TABLE II. Relationship between Litter Series, 14th Day Weight of Young and Number of "Functional" Mammary Glands.

Position of litter series No. of females	I 89	II 59	III 39	IV 34
Avg No. of functional glands	9.6	10.2	10.3	10.2
S.E.	0.12	0.17	0.17	0.20
Avg wt of 6 young on 14th day (g)	122.5	137.5	143.1	138.3
S.E.	1.59	2.30	2.69	3.14
Avg birth wt of 6 young (g)	29.35	30.28	31.38	30.71
S.E.	0.223	0.173	0.696	0.243

ter was the lowest as compared to the weights of those of the subsequent series. The average 14th day weight of 6 young born in the third order of birth was the highest. It is interesting to note that the average number of "active" mammary glands in the females of the first group was the least. It may seem, therefore, that there is a correlation between the number of "active" mammary glands and the position of litter series.

Discussion. The wide variation in the weights of young rats from different litters has been mainly due to the maternal milk supply. A closer study revealed that there was a linear relationship between the number of "milking" mammary glands of the mother rats and weights of their offspring. Actually it is possible to explain on the basis of the number of "active" or "milking" mammary glands the observed differences in the weaning weights of young born in the different order of birth. It should, however, be admitted that a corresponding study of milk yield of the lactating mothers would have been of considerable importance, but certain technical difficulties did not permit of extensive studies being made on this aspect.

Summary. An attempt has been made to determine the cause(s) for variation in weight of suckling rats. It was found that the 14th day weight of rats did depend upon the number of "milking" glands of the mother rats. The average 14th day weight of young born in the first litter was lowest when compared with the weights at corresponding periods of those delivered in the subsequent litters. The average number of "functional" mammary glands of mothers nursing their first born was also the lowest as compared with those of mothers rearing their second, third and fourth litters.

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1. Schultze, M. O., *J. Nutr.*, 1954, v54, 453.
2. Hubbell, R. B., *ibid.*, 1954, v53, 429.

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Essential Fatty Acids in the Chick. I. Development of Fat Deficiency.*

(22718)

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Symptoms of essential fatty acid deficiency in various animal species in which it has been produced are rather similar. Thus, in the rat(1), dog(2), guinea pig(3), and mouse (4), onset of deficiency is relatively slow and becomes apparent in changes in skin and fur as well as in decreased growth rate. The periods required for depletion of essential fatty

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* Preliminary report appeared in *Poultry Sci.*, 1955, v34, 1231.

TABLE I. Composition of Purified Diet.

Ingredient	g/kg
Purified casein[*]	200
Gelatin	80
Glucose (cerelose)	615
Corn oil[†]	40
DL-methionine	3
Salts A(6)	60
Vitamins[‡]	2
Aureomycin[§]	

[*] General Biochemicals, Inc.

[†] Mazola.

[‡] Vitamins added as follows (in cerelose premix), mg/kg diet: Vit. B_1 8, vit. B_2 8, calcium pantothenate 20, choline chloride 2000, niacin 100, vit. B_6 8, d-biotin 0.3, folic acid 3, vit. B_{12} 0.02, vit. D_3 0.02, α-tocopherol acetate 100, and 2-methyl-1,4-naphthoquinone 1. Vit. D, E, and K added in ethanol. 30 mg/kg of vit. A acetate added in ethanol in first exp. 1 mg vit. A acetate given orally, weekly, in 2nd and 3rd exp. See text.

[§] 200 mg/kg diet added after 8 wk of 2nd and 3rd exp.

acids, as detected by these symptoms, are 4 weeks in the mouse and guinea pig, 8-12 weeks in the rat, and 12-40 weeks in the dog. Morbidity and death in these animals occur considerably later. It is apparent that tissues of these species relinquish their polyunsaturated fatty acids at a slow rate. In contrast, it has been reported that chicks on a synthetic-type, fat-free diet grew slowly from the first week, feathered poorly, and died within 3 to 4 weeks(5). These observations suggest that the chick has a considerably greater essential fatty acid requirement than other species. Contrary to this viewpoint, our studies indicate that chicks grow normally on a fat-free diet for at least 6 to 8 weeks, and do not develop symptoms of deficiency until later.

Methods. Day-old female New Hampshire chicks in groups of 6 were placed in electrically heated brooders with screen mesh floors and fed the synthetic-type diet given in Table I, with or without inclusion of 4% corn oil. Both feed and water were given *ad lib.* The air-conditioned room was kept at 75 ± 1° F and 55% relative humidity. In initial experiments it was found that 3 mg of vit. A acetate/kg, mixed into the diet in ethanol, was so rapidly destroyed (as found by analysis) that vit. A deficiency frequently developed in the chicks in 3-4 weeks. This could be prevented by increasing the level to 30 mg/kg. However, occasional appearance of vit. A deficiency symptoms even under these conditions prompted a study of parenteral and oral administration of this vitamin. A single, weekly oral dose of 1 mg vit. A acetate in 0.4 ml of 20% aqueous Tween 40[†] gave a sufficiently high liver storage of the vit. to prevent deficiency. Chemical analyses of livers from chicks in these experiments showed that both vits. A and E were absorbed in adequate amounts. Chicks were weighed and examined weekly. When 8 weeks old, the groups were reduced to four birds each because of space limitations.

Results. In preliminary experiments lasting 4 weeks, birds on fat-free diet grew as well as those receiving 4% corn oil in the diet and appeared normal in all respects as indicated in a preliminary report(7). Experiments of longer duration were then carried out and growth data from 3 separate trials are averaged in Table II. Although control chicks were slightly heavier throughout, the difference at 8 weeks was not significant $(P = >0.1)$. By 12 weeks chicks fed the fat-free diet were definitely smaller $(P = <0.01)$. It was apparent from the growth curves that effects of fat-deficiency appeared between the 6th and 8th weeks. The first visible symptom attributed to fat deficit was a lightening of normally dark brown plummage, occurring from about the 8th week. This was not marked and did not progress or occur uniformly in all birds. Changes in skin were not readily detectable. In several birds an increased scaliness occurred after 16-20 weeks.

Of the 8 fat-deficient chickens kept beyond 8 weeks, 4 were maintained as long as 24 weeks. Three others died at 13, 22 and 24 weeks, and one was killed at 17 weeks. Throughout this period the appearance of the birds was normal except for the slight changes noted above. Death of chicks in the first trial was attributed to air sac infection. In succeeding experiments 200 mg aureomycin/kg of diet was fed after the birds were 8 weeks of age and this cause of death was eliminated.

[†] Polyoxyethylene sorbitanmonopalmitate.

TABLE II. Growth of Chicks Fed a Fat-Free Diet (Avg of 3 Trials).*

	Fat-free diet			Control diet		
Age (wk)	Wt in g	No. surviving	Feed effic.†	Wt in g	No. surviving	Feed effic.
4	295 ± 11‡	18	.549	303 ± 12	18	.537
6	560 ± 13	17		579 ± 16	18	
8	830 ± 26	17		894 ± 30	16	
12	1288 ± 46	8*		1512 ± 49	8*	

* Six chicks/group in each trial. Only 2 trials were continued for 12 wk and longer; the No. of chicks was reduced to 4 in each group after 8 wk.

† Total wt gain/total food consumed.

‡ Mean ± S.E.

In two birds which died in later experiments, no obvious cause of death could be detected. At autopsy, fat-deficient chickens (24 weeks) had considerable amounts of abdominal fat, though less than controls. The oviducts and ovaries in two birds were immature, but in the other two the ovaries contained developed yolks. All other organs appeared normal.

Three male chicks maintained on fat-free diet for 8 weeks had combs and wattles which were smaller and paler than those of three males receiving 4% corn oil in the diet. At autopsy, the testes of fat-deficient chicks were only half as large as those of control chicks (178 vs. 441 mg/kg body weight). Body weights of deficient birds were only slightly less (6%) than those of normal birds.

Water consumption measurements made during the ninth week revealed that fat-deficient birds drank only about 15% more than the controls. In two trials made during the 11th and 12th weeks deficient chicks consumed 137 ml/kg/day compared with 103 ml/kg/day for normal chicks (4 per group).

Discussion. These experiments show that when chicks are fed a synthetic-type diet devoid of fat but adequate with respect to all other nutrients, growth continues normally for at least six weeks. Furthermore, the symptoms of fat deficiency that eventually develop are not striking. These results are in marked contrast to the picture presented by Reiser(5), and which has been quoted in recent reviews(8,9), that chicks grow poorly and die within one month when deprived of fat. It would appear that the diet used by Reiser was probably deficient in nutrients other than fat. The difficulty in insuring a sufficient intake of vit. A in a fat-free diet has already been discussed above. In addition to a possible vit. A deficiency, the diet of Reiser may also have been low in thiamine since the soybean protein, used in his diets, contains sulfite which has been shown to destroy thiamine(10). The salts V, which he used, accelerate thiamine destruction(11). Repetition of Reiser's experiments in our laboratory, using egg albumen as the protein but with vit. A given orally as described above, did not result in any difference between control and fat-free birds at 4 weeks

Analysis of purified casein(12) used in these experiments revealed a fat content of 0.23% and examination of the polyunsaturated fatty acids(13) in this lipid showed only 4.4% of dienoic and no trienoic or tetraenoic acid. This would amount to an intake of less than one mg dienoic acid daily (0.046% lipid in the diet). In comparison with the linoleic acid requirement of other species, this amount would be negligible. This same casein in a similar purified diet readily produces essential fatty acid deficiency in the guinea pig(3).

The decrease in growth rate, which occurred after 6-8 weeks on the fat-free diet, was also found by Russell *et al.*(14), although their difference began at 10 weeks and was not as marked. The higher caloric content, or an increased palatability of the control diet with fat, may have contributed to these differences. In the present studies feed efficiencies of the control and fat-free birds were found to be similar at 4 weeks (Table II).

Summary. Day-old chicks raised on a purified diet containing approximately

0.046% fat grew normally for 6-8 weeks, at which time weight gains began to decrease slightly. No marked symptoms of deficiency developed, but depigmentation of feathers and scaliness of skin were observed. Fat-deficient male chicks had smaller testes and some females had immature oviducts, when compared with normal chicks. Water consumption by the deficient birds did not increase markedly until the 11th week on the fat-free diet.

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1. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1929, v82, 345.

2. Hansen, A. E., and Wiese, H. F., *Texas Rept. Biol. Med.*, 1951, v9, 491.

3. Reid, M. E., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 708.

4. White, E. A., Foy, J. R., and Cerecedo, L. R., *ibid.*, 1943, v54, 301.

5. Reiser, R., *J. Nutrition*, 1950, v42, 319.

6. Briggs, G. M., Spivey, M. R., Keresztesy, J. C., and Silverman, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 113.

7. Briggs, G. M., and Fox, M. R. S., *Poultry Sci*, 1955, v34, 1231.

8. Holman, R. T., *The Vitamins*, vol. II, W. H. Sebrell and R. S. Harris, ed., Academic Press, New York, 1954, 296.

9. Deuel, H. J., Jr., and Reiser, R., *Vitamins and Hormones*, vol. XIII, R. S. Harris, G. F. Marrian and K. V. Thimann, ed., Academic Press, New York, 1955, 43.

10. O'Dell, B. L., Boston, M. D., Savage, J. E., and Hogan, A. G., *Poultry Sci.*, 1952, v31, 559.

11. Waibel, P. E., Bird, H. R., and Baumann, C. A., *J. Nutrition*, 1954, v52, 273.

12. Assn. Off. Ag. Chem., *Methods of Analysis*, 1955, 210.

13. Wiese, H. F., and Hansen, A. E., *J. Biol. Chem.*, 1953, v202, 417.

14. Russell, W. C., Taylor, M. W., and Palskin, L. J., *J. Nutrition*, 1940, v19, 555.

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Variations in After-Discharge from the Hippocampus. (22719)

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A number of investigators have noted the relative sensitivity of the hippocampal formation to the production of after-discharge(2,5, 8,9). It has also been noted that the after-discharge shows little or no spread to the rostral neocortex(3), although there is some disagreement on the extent of spread. Akert and Andy(1) describe the local after-discharge in the guinea pig as characterized by 18-25 c/sec. regular waves with occasional activity at slower or faster frequencies. Liberson and Akert(6) describe a tonic-clonic sequence. The tonic phase consists of 10-30 c/sec. activity associated with brief spikes of 100-250 c/sec. frequency. The relation of the DC potentials, described earlier by Liberson and Cadilhac(7), to the clonic phase was indicated. Crutzfeldt and Meyer-Mickelett(3), using cats, describe 3 characteristic phases preceded by a period of 50-100 c/sec. activity. The first phase consists of 8-12 c/sec. waves and is followed by 18-28 c/sec. activity. The final phase consists of short pauses and single spikes.

The purposes of the present study were to determine the components of hippocampal after-discharge in the cat, and more specifically the different patterns which these components may form. A study was also made of variations in after-discharge threshold within the hippocampal formation, and an attempt was made to relate variations in pattern of after-discharge to a number of antecedent conditions.

Material and methods. Bilateral bipolar electrode placements were made in a series of 20 cats with the aid of a Horsley-Clark stereotaxic instrument. All operations were performed under aseptic conditions using sodium pentobarbital (Nembutal) anesthesia. In all cases placements were as nearly symmetric as possible, and at the level of the posterior portion of the lateral geniculate body. Some variation was introduced in the vertical plane to cover different structures within the hippocampal formation. The bipolar electrodes were nichrome steel, insulated to within one millimeter of the tips, and placed with the tips one-half to one millimeter apart. Small stainless steel screws were placed in the skull over the middle suprasylvian gyrus to serve as cortical recording electrodes. All recording was done with a Grass Model III-D Electroencephalograph. Stimulation was by means of a Grass Model 3C Stimulator and isolation unit. Stimulation was with diphasic pulses of one millisecond duration at a rate of 80 pulses per second (pps.). To study the effects of stimulus frequency rates of 20 pps. and 300 pps. were also used. No records were taken for several days following operation to allow complete recovery of the animal. After-discharge was produced by stimulating the hippocampal formation with the stimulus parameters described for 10 seconds at intensities of 1 to 5 volts. In studies on effects of voltage the duration of stimulus was reduced to 5 seconds to minimize tissue destruction at high voltages. The duration of the after-discharge periods ranged from 2 or 3 seconds following termination of stimulus to just under 2 minutes. Most of them lasted between 30 and 40 seconds. For analysis of the patterns of after-discharge, only those which persisted long enough for development of a clear pattern were used.

Analysis of the after-discharge activity is based on 91 seizure records of 16 cats. Studies of the effects of voltage, pulse rate, time between seizures and mode of stimulation (ipsilateral, bilateral or contralateral) were carried out on 4 additional cats. The right hippocampal tracing was the one analyzed in all cases. For bilateral stimulation the right and left hippocampal bipolar electrodes were connected in parallel. Voltage was monitored at all times by means of a cathode ray oscilloscope. Histological control was available for all animals.

Results. The most frequently seen components were spikes of varying duration. They tended to fall in bands of 15-20 milliseconds, 30-40 milliseconds and 60-80 milliseconds. Occasionally faster spikes were visible, but their characteristics could not be adequately determined with an inkwriter. The slower spikes were seen more frequently than the faster and ranged in amplitude from 1 to 2 millivolts. The faster spikes were usually at lower voltage. These faster spikes typically were diphasic or triphasic in form and characteristically ranged from 100 to 600 microvolts in amplitude. Slower waves, more sinusoidal in form, were seen at a frequency of 5-11 c/sec., and occasionally activity at ½ to 4 c/sec. was present. Rhythmic activity between 20 and 30 c/sec. was at times seen for brief periods. It was most often present at 26 c/sec., but also frequently at 22 c/sec.

These components tend to become organized into several relatively discrete patterns. Five such patterns were empirically defined on the basis of wave form, frequency and voltage of the predominant activity. Examples of these groups are shown in Fig. 1. Group 1 was defined on the basis of a low to moderate voltage (100 to 400 microvolts peak to peak) 1½ to 9 c/sec. rhythmic activity. The slower activity was usually at the lower voltages. This activity may occur alone, but often the shift across the baseline is accompanied by a burst of fast low voltage spikes. Occasionally this activity may occur with a near normal tracing superimposed on it. In some records the spikes bear a constant relationship to the slower wave, but often they do not. Rarely a classical "wave and spike" pattern was seen.

Group 2 was the largest group, and 12 of the 16 animals had records falling in this group. It includes those cases where the predominant activity is spikes. The slower spikes were more highly organized and occurred at a repetition rate of 7½ to 12 per second, but most frequently at 9 per second. These spikes are monophasic and occur on the crest of a low voltage (50 to 100 microvolts) slow wave. The faster spikes are more complex, as previously indicated, and also occur with slower waves of low voltage and rhythmic 20 to 30 c/sec. activity. These records tend to show more components with more variable relationships between them than do the records with slower spikes.

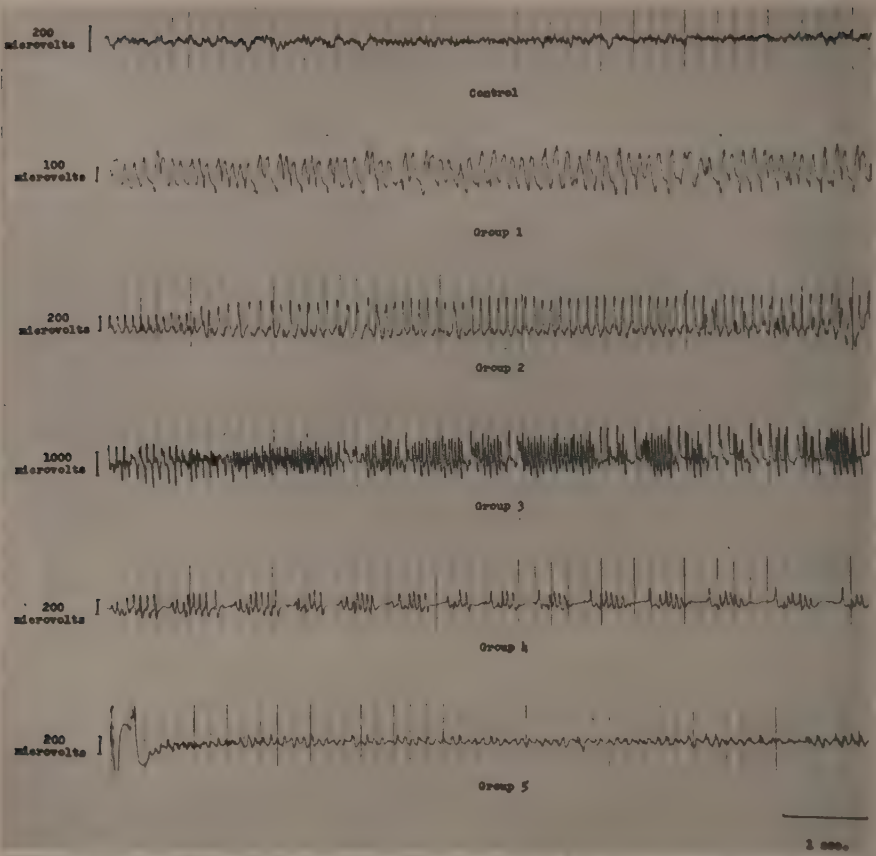

FIG. 1. Patterns of after-discharge obtained by stimulation of the hippocampal formation, and a representative section of normal hippocampal activity. These records were taken from different cats.

Group 3 is relatively rare, and was seen in only 3 of the 16 animals. It was defined on the basis of a predominant rhythmic fast activity ranging from 20 to 30 c/sec., but most often seen at 26 c/sec. Sharp spikes and slow waves are the most usual accompanying activity. Group 4 was defined on the basis of an alternating burst and depression. Most typically the bursts are made up of 4 or 5 15-30 c/sec. sharp waves which are separated by a silent period. This pattern was seen in 7 of the 16 cats. Group 5, seen in 5 of the 16 cats, was defined on the basis of a low voltage continuous rhythmic activity at frequencies of 5-15 c/sec. This may be mixed with low voltage fast spikes. More than one of these patterns may appear during a single seizure, though one is nearly always predominant. If the records are grouped on the basis of the predominant pattern, records from the same cat fall into several groups. The range was from 1 to 4 with a median of 2.5.

Fig. 2 illustrates a number of the characteristics of hippocampal after-discharge when the entire period of after-discharge is considered. When the after-discharge is compared with the control record, the absence of any great disturbance in the dorsolateral cortex is apparent. The pattern of after-discharge on the right and the left shifts markedly (more than is typical) throughout the course of the seizure, and the activity on

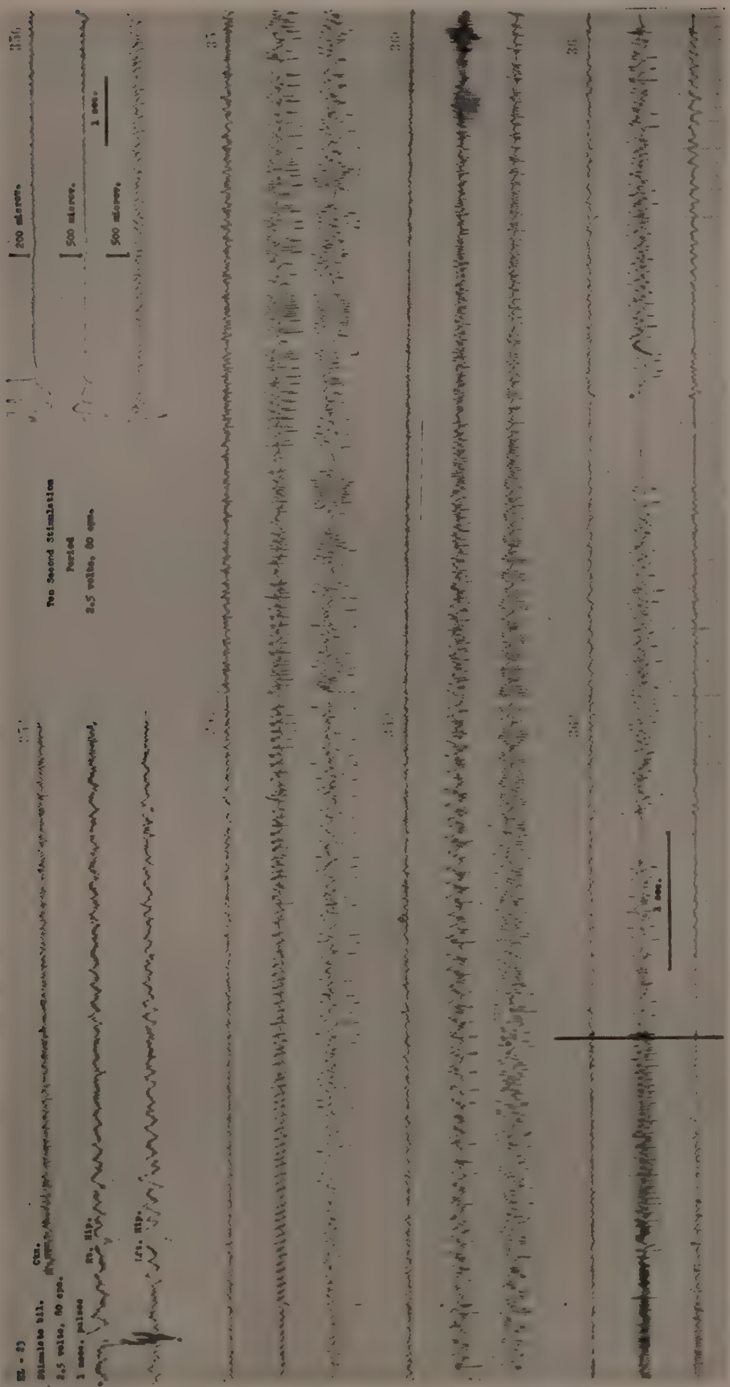

FIG. 2. A complete record of hippocampal after-discharge obtained by bilateral stimulation at 2.5 volts, 80 pps. See text for discussion.

the two sides is almost never identical. However, the brief silent periods, when they occur, usually occur simultaneously on the right and the left. Shifts in after-discharge form nearly always occur simultaneously in the 2 hippocampi. The long build-up which is present on the right is frequently seen, and may not be equal bilaterally. The gradual ending of the seizure activity on the left is atypical, as the seizures usually end quite abruptly and simultaneously on both sides. If there is a difference in the voltage of the after-discharge on the right and the left, the higher voltage is usually on the side stimulated. There is usually a brief period of depressed electrical activity following a seizure. In rare instances random spikes persisted for several minutes following a seizure, and appeared independently on the right and the left.

Of the locations investigated, the dentate fascia had the lowest thresholds for the production of after-discharge and the subiculum the highest. In some cases after-discharge could not be elicited from the subiculum. The alveus also has a high threshold in the only 2 placements in that location, as did the ventromedial edge of the hippocampus. In general, the fiber tracts running between the pyriform and retrosplenial areas and the hippocampus yielded an intermediate threshold.

Since the pattern of after-discharge was not dependent on the location of the electrodes, at least entirely, other factors were investigated. When the patterns were ranked by mean threshold and by mean time since operation there was a statistically significant relationship between the rankings ($r = .77$). Threshold means were computed by finding the overall mean for each cat and giving each record a value in terms of deviation from this mean. These deviation values were then averaged for each pattern. Group 4 showed the lowest threshold and Group 2 the highest. There was very little difference between the stimulus intensities required to produce after-discharge at 20, 80 and 300 pps., but 80 pps. seemed slightly more effective (not statistically significant). There was no evidence that any of the components of after-discharge, or the patterns they formed was related to the stimulus frequency, or to the voltage used provided it was above threshold. There was consistent evidence of reduced excitability two minutes following the end of a seizure. Occasionally longer intervals were tested, but the results were inconsistent.

Discussion. The results disclose considerably more variability in the form of the after-discharge from the hippocampal formation than has previously been indicated, although the components seen confirm those previously reported for the guinea pig(1,5) and the cat (4,8). The sequences of activity reported by Liberson and Akert(6), and by Crutzfeldt and Meyer-Mickelett(3) were both seen, but were no more frequent than other sequences noted. The complete tonic-clonic sequence was rather rare. The gradual build-up frequently seen would seem to indicate a recruitment process within the hippocampus. The fact that quite different patterns of after-discharge could be recorded from the same electrode implant with identical stimulus parameters would strongly suggest that the nature of the recorded activity is dependent upon the state of the organism at the time the stimulus is applied.

Attempts to determine the preceding conditions responsible for the variations in form of the after-discharge have not been very successful. The fact that there was a relationship between threshold and pattern suggests that the level of excitability at the time the stimulus is applied may be one significant variable. This is further supported by the significant relationship between threshold and time since operation for the different patterns, and would suggest some progressive modification of the hippocampus as one determining variable. If elements within the hippocampus are differentially affected by previous stimulation, then certain variations in pattern could represent the absence of participation of some elements. Additional work is necessary on this aspect.

Conclusions. The after-discharge of the hippocampus is formed by a number of components which can combine to form several fairly discrete well organized patterns. These patterns seem to be related more to the state of the organism at the moment the after-discharge is produced than to the precise anatomic location of the electrodes or to the parameters of stimulation. Although the activity emanating from symmetrical electrode placements may show differences in pattern, there are systematic relationships between the right and the left which would indicate some bilaterally expressed control on the after-discharge. Extremely high voltage electrical after-discharge may be present in the hippocampal formation with only minimal disturbance in the dorsolateral cortex, at least by transverse recording, and without the precipitation of a major convulsion.

1. Akert, K., and Andy, O. J., *Trans. Am. Neurol. Assn.*, 1953, 194-195.

2. Andy, O., and Akert, M., *EEG Clin. Neurophysiol.*, 1953, v5, 320.

3. Crutzfeldt, O. D., and Meyer-Mickelett, R. W., *EEG Clin. Neurophysiol.*, 1953, Suppl. III, 43.

4. Green, J. D., and Shimamoto, T., *Arch. Neurol. Psychiat.*, 1953, v70, 687.

5. Liberson, W. T., and Akert, K., *EEG Clin. Neurophysiol.*, 1953, v5, 320.

6. ———, *ibid.*, 1955, v7, 211.

7. Liberson, W. T., and Cadilhac, J. G., *ibid.*, 1953, Suppl. III, 42.

8. MacLean, P. D., *J. Neurosurg.*, 1954, v11, 29.

9. Morin, F., and Green, J. D., *Anat. Rec.*, 1953, v115, 433.

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The Effect of Molybdenum on Chick and Poult Growth. (22720)

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The ash of unidentified factor sources has been reported to stimulate the growth of both chicks and poults when supplemented to a purified type diet(1,2). The ash of dried whey, fish solubles or distillers dried solubles has also been found to stimulate the growth of chicks fed a practical type diet under field conditions(3).

Single additions of 40 elements to a purified diet for chicks were ineffective in duplicating the growth response obtained with the ash of a mixture of 6 unidentified factor sources(8). A reconstituted ash mixture, which was made up of chemically pure, reagent grade chemicals and supplied the elements found in the ash of distillers dried solubles upon spectrographic analysis, stimulated the growth rate of both chicks and poults (4). A similar reconstituted ash mixture produced a growth response under practical conditions (3).

Molybdenum has been found to be a cofactor of xanthine oxidase in the rat(5,6). The maximum molybdenum required by the rat for xanthine oxidase activity has been estimated as 0.2-0.3 μg per day(7). However, the growth rate of rats receiving the diets low in molybdenum was not affected. Molybdenum deficiency in the chick was associated with the feeding of 4.5-9.4 mg % tungsten in the ration(9). Such a deficiency could be overcome through addition of molybdate to the diet. The deficiency was characterized by growth depression, mortality, lowered tissue molybdenum concentrations, reduced xanthine dehydrogenase activity in the liver, intestine, kidney and pancreas, and decreased uric acid excretion.

Methods. Chicks were obtained from dams which had been reared from one day of age on an all-vegetable protein diet deficient in unidentified growth factors. The maternal diet used was the same as previously reported by Dannenburg *et al.*(1). Two types of chicks were used, New Hampshire and a New Hampshire X Barred Plymouth Rock Cross.

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TABLE I. Basal Diets.

Ingredients	Chicks	Poults
	g/kg	
Cerelose	625.7	528.8
Soybean protein (Drackett C-1)	250	325
Soybean oil	30	35
$CaCO_3$	14.5	16.03
$CaHPO_4 \cdot 2H_2O$	42.1	60.10
NaCl	9.5	5
$MnSO_4 \cdot 1\,H_2O$	.78	.81
$FeC_6H_6O_7 \cdot 5H_2O$	1.6	1.60
$CuSO_4 \cdot 5H_2O$	.016	.02
$ZnCl_2$	.014	.014
KI	.040	.040
$CoCl_2 \cdot 6H_2O$	.0005	.0005
KCl	5.72	6
$MgSO_4 \cdot 7H_2O$	5.7	5.76
	Amt/kg	
Thiamin HCl	4 mg	5 mg
Riboflavin	6	8
D-Calcium pantothenate	15	16
Pyridoxine HCl	4	4
Para-Aminobenzoic acid	20	20
Alpha-tocopheryl acetate	24	24
Inositol	1,000	1,000
Menadione	.5	.5
Biotin	.2	.2
Niacin	100	120
Choline chloride	2,000	2,000
Folic acid	2	2
Aureomycin HCl	10	50
Vitamin A	10,000 I.U.	10,000 I.U.
" D_3	2,000 I.C.U.	2,000 I.C.U.
" B_{12}	30 μg	50 μg
DPPD	125 mg	125 mg
DL-methionine	7.5 g	7.5 g
Glycine	4 g	4 g

The Broadbreasted Bronze poults were obtained from dams which had not been depleted of unidentified factors. The birds in each experiment were distributed at random into groups of 10 to 16 birds each, and in the case of the chicks each treatment was replicated once. Four replicates were used on the basal ration in the chick studies and two in the poult studies conducted. The birds were maintained in batteries with raised wire floors throughout the four-week experimental periods. Feed and water were supplied *ad lib.*

The basal diets used are shown in Table I. The diets for both the chicks and poults have been checked for adequacy by feeding higher protein levels, a combination of the 11 essential amino acids at levels of 0.1 of the N.R.C. recommended levels, additional vitamins as well as double and four times the trace minerals without producing additional growth. The soybean protein (Drackett C-1) was extracted with either 70% isopropanol or hot water (pH 4.6) in chick diets in order to remove any unknown growth factors associated with this dietary component. The ash of distillers dried solubles was prepared by ignition over an open flame followed by ashing in a muffle furnace at 600°C until a constant weight was obtained (8 to 10 hours). The composition of the reconstituted ash mixture used which consisted of the elements present in the ash upon spectrographic analysis is shown in Table II.

Results. Feeding molybdenum (0.0126 ppm) in a purified diet for chicks resulted in an 18.7% increase in growth (Exp. 1, Table III). In the same experiment, distillers dried solubles produced an increase in growth of chicks to 4 weeks of 97 g over the unsupplemented group. Approximately one-half of the growth response obtained with distillers dried solubles was produced by feeding the ash of this product at a level equivalent to 3%. The reconstituted ash mixture was equivalent to distillers dried solubles ash in growth-promoting properties (Table III).

In the study using crossbred chicks, a 13.5% growth increase was obtained through addition of 0.0126 ppm of molybdenum to the basal diet. This response was found to be significant at the 0.05 level of probability. The reconstituted ash mixture in this experiment produced an 11.4% increase in growth while the higher level of molybdenum (3.3 ppm) produced a growth response of 9.7%. Both of these growth rates were significantly greater than that of the unsupplemented control group (Table III).

Using Broadbreasted Bronze turkey poults, a 29.1% growth response was obtained with reconstituted ash mixture fed at a level equivalent to 6% distillers dried solubles. Inclusion of 0.0126 ppm of molybdenum in the basal diet produced a 19.1% increase in growth rate.

It is interesting to note that growth responses obtained with molybdenum in these

TABLE II. Composition of Reconstituted Ash Mixture.

Ingredient	Formula	Amt equivalent to 100 g ash* mg	g
Aluminum sulfate	$Al_2(SO_4)_3 \cdot 18\,H_2O$	370.8	
Barium hydroxide	$Ba(OH)_2 \cdot 8H_2O$	13.7	
Sodium borate	$Na_2B_4O_7 \cdot 10H_2O$	264.0	
Chromic oxide	Cr_2O_3	1.46	
Cobalt chloride	$CoCl_2 \cdot 6H_2O$	4.04	
Copper sulfate	$CuSO_4$ (anhydrous)		3.93
Ferric phosphate	$FePO_4 \cdot 4H_2O$		12.0
Magnesium sulfate	$MgSO_4$ (anhydrous)		3.04
Manganese chloride	$MnCl_2 \cdot 4H_2O$		1.08
Molybdic acid	$H_2MoO_4 \cdot H_2O$	56.26	
Nickelous carbonate	$NiCO_3 \cdot 2Ni(OH)_2 \cdot 4H_2O$	64.08	
Ca. phosphate (dibasic)	$CaHPO_4 \cdot 2H_2O$		7.33
Potassium chloride	KCl		5.73
Sodium meta-silicate	$Na_2SiO_3 \cdot 9H_2O$		3.04
Sodium chloride	$NaCl$		7.63
Strontium chloride	$SrCl_2 \cdot 6H_2O$	304	
Titanium oxide	TiO_2	5	
Zinc chloride	$ZnCl_2$	62.53	
Lead nitrate	$Pb(NO_3)_2$	159	
Silver nitrate	$AgNO_3$	47.2	

* 451.3 mg of reconstituted ash mixture were equivalent to 1 g of distillers dried solubles ash.

studies were equivalent to those obtained with either distillers dried solubles ash or reconstituted ash mixture in the chick experiments, while the results of the poult study indicate an added growth advantage through the feeding of reconstituted ash at a higher level. These data would indicate a higher requirement for molybdenum in the turkey poult under the conditions of these studies. The molybdenum contents were determined to be 0.972 ppm and 1.58 ppm, respectively, for the chick and poult diets. It is difficult to understand how inclusion of only 0.0126 ppm of molybdenum in these diets would produce an increase in growth, unless a large portion of the molybdenum present in the basal rations was unavailable. Richert and Westerfeld(7) have indicated that the molybdenum in soybean oil meal was largely unavailable to the rat. The molybdenum content of soybean protein (Drackett C-1), before alcohol or water extraction, was found to be 4.77 ppm; while the extracted protein contained 3.84 ppm of molybdenum. If it were assumed that

TABLE III. Effect of Minerals on Chick Growth.

	Exp. 1 (New Hampshire chicks)		Exp. 2 (New Hampshire × Barred Rock)		Exp. 3* (Broadbreasted Bronze poults)	
Supplements to basal diet	4-wk avg wt (g)	Response over basal (%)	4-wk avg wt (g)	Response over basal (%)	4-wk avg wt (g)	Response over basal (%)
None	289.7	—	346.1	—	403	—
3% distillers dried solubles	376.7‡	26.2	—	—	—	—
Distillers dried solubles ash equivalent to 3%	330.3†	14.0	—	—	—	—
Reconstituted ash equivalent to 3% distillers dried solubles	335.2†	15.7	385.6†	11.4	520‡	29.1
Molybdic acid, 0.0126 ppm Mo	344.0†	18.7	399.9†	13.5	480†	19.1
" " 3.3 " "	—	—	379.8†	9.7	—	—

* The reconstituted ash was fed at level equivalent to 6% Distillers Dried Solubles.

† Significant at 0.05 level of probability.

‡ " " 0.01 " " ".

molybdenum in the protein source was unavailable to the chick, the basal diet would contain only 0.0228 ppm of "available" molybdenum supplied by the mineral mixture. However, this remains to be proven. The turkey mineral mixture contained 0.3 ppm of molybdenum and supplied 0.0287 ppm to the basal ration. It would appear that both the chick and poult have a nutritional requirement for molybdenum under the conditions of these studies.

Summary. 1) The results of 2 studies with chicks and one study using Broad Breasted Bronze turkey poults have indicated that addition of molybdenum to purified basal diets resulted in a growth advantage of 13.5-19.1% over the unsupplemented group. 2) The feeding of a reconstituted ash mixture which simulated the ash of distillers dried solubles resulted in significant increases in growth rate of both chicks and poults. 3) From the data presented it appears that under conditions of these studies a nutritional requirement for molybdenum by chick and poult may be demonstrated.

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1. Dannenburg, W. N., Reid, B. L., Rozacky, E. E., and Couch, J. R., *Poultry Sci.*, 1955, v34, 1023.
2. Morrison, A. B., Scott, M. L., and Norris, L. C., *ibid.*, 1955, v34, 738.
3. Camp, A. A., Reid, B. L., and Couch, J. R., *ibid.*, 1956, v35, 621.
4. Reid, B. L., Rozacky, E. E., and Couch, J. R., *ibid.*, 1955, v34, 1217.
5. de Renzo, E. C., Kaleita, E., Heytler, P. G., Oleson, J. J., Hutchings, B. L., and Williams, J. H., *J. Am. Chem. Soc.*, 1953, v75, 753.
6. Westerfeld, W. W., and Richert, D. A., *J. Biol. Chem.*, 1953, v203, 915.
7. de Renzo, E. C., Kaleita, E., Heytler, P. G., Oleson, J. J., Hutchings, B. L., and Williams, J. H., *Arch. Biochem. and Biophysics*, 1953, v45, 247.
8. Morrison, A. B., Dam, R., Norris, L. C., and Scott, M. L., *Fed. Proc.*, 1956, v15, 564.
9. Higgins, E. S., Richert, D. A., and Westerfeld, W. W., *Fed. Proc.*, 1956, v15, 274.

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4,4′-Bisacetamidocarbanilide, a Metabolite of Nicarbazin. (22721)

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Nicarbazin, a chemical complex of 4,4′-dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethyl pyrimidine (HDP), is an effective and widely used anticoccidial agent for chickens(1,2). After nicarbazin is absorbed by chickens, the complex separates into its components, which are rapidly eliminated from the tissues(3). Clark, *et al.*(4) used nicarbazin labelled with C^{14} in the benzene ring of the DNC to determine if metabolites, which were undetected by chemical assay, remained in the tissues. These studies showed that most radioactivity found in tissues of chickens fed or dosed with C^{14} labelled nicarbazin was accounted for as colorimetrically assayable DNC. Although traces of metabolites of DNC were present in tissues of chickens, they were rapidly destroyed or excreted.

During the performance of tolerance studies with large doses of nicarbazin(5), crystalline deposits were noted in the renal tubules of calves. Therefore, studies were conducted to isolate this material, which was identified as 4,4′-bisacetamidocarbanilide, a metabolite of nicarbazin.

Methods. Nicarbazin was fed to 8-10-week-old calves for 4 to 6 weeks. The nicarbazin, in daily dosages of 0.04, 0.2 or 1.0 g/kg, was suspended in about one-half of the calves' morning milk ration. When this was consumed, the balance of the milk was fed. Water, hay and a commercial calf-starter were fed *ad lib*.

Results. The calf on the lowest dosage of nicarbazin (0.04 g/kg) consumed 93 g of the drug during 6 weeks of administration. This calf gained in weight from 108 to 167 lb, appeared normal and maintained a good appetite. When this calf was sacrificed at end of dosage period, all organs were grossly and histologically normal. Another calf was fed a total of 565 g of nicarbazin in a daily dosage of 0.2 g/kg. During the first week of administration, this calf lost 10 lb in weight but afterwards gained slowly, increasing in weight from 153 to 183 lb. Although this calf continued to have a good appetite and appeared normal clinically, at autopsy the kidneys were enlarged and showed evidence of damage. The calf fed nicarbazin at the rate of 1 g/kg received 1524 g of the compound. Failure to gain in weight was evident during the first week of administration. During the next 3 weeks this calf lost 34 lb in weight, refused to eat normally, weakened and died. Necropsy examination showed the kidneys were enlarged. The cortex was pale and firm. The medulla revealed gray streaks next to the cortex and in its inner portion there were groups of minute tan-colored foci suggestive of crystalline deposits.

Histopathological examinations were made on the myocardium, aorta, bone marrow, lymph nodes, thymus, thyroid, lungs, pancreas, stomach, intestines, urinary bladder, adrenals, striated muscle, liver and kidneys. The only significant pathologic alterations were in the liver and kidney. The liver lesion was found only in the calf fed the highest dosage of nicarbazin (1 g/kg). The lesion was confined to the portal area and consisted of a slight to moderate bile duct proliferation and fibrosis. The kidney changes included precipitation of crystals (Fig. 1) mainly in the distal portions of the collecting tubules and a few in the convoluted tubules, probably the distal ones. The tubular epithelium directly adjacent to the crystals showed hyperplastic changes consistent with a foreign body reaction. The cortex was characterized by a focal, granulomatous, interstitial reaction. Within these areas of the cortex, hyperplastic changes were present in the tubules and small blood vessels, even in the apparent absence of crystals. Some tubules were filled with neutrophilic leucocytes and eosinophilic casts. Degenerative changes were noted in other convoluted tubules.

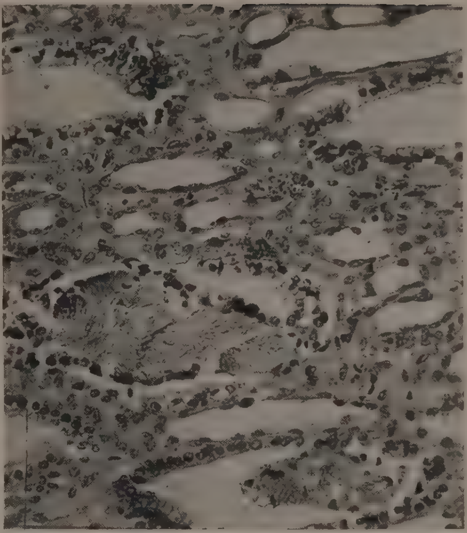

FIG. 1. Crystals of 4,4′-bisacetamidocarbanilide in collecting tubules of calf kidney.

To isolate and identify the crystalline material, portions of the kidneys were ground with a meat chopper and digested overnight at 37°C in artificial gastric juice. After straining and sedimenting, the material was washed with distilled water and centrifuged several times until the supernatant was clear. Microscopic examination of the sediment showed that considerable fine tissue debris was present. Therefore, the crystalline material was suspended briefly in normal NaOH solution, centrifuged and repeatedly washed with several changes of distilled water. The crystals were assayed for DNC but a negative test was obtained.

The crystalline material was gray in color, insoluble in water, dilute acid and the common organic solvents. Likewise in dilute alkali the solubility was essentially nil. It burned somewhat sluggishly and left a slight residue which exhibited a typical brick-red color characteristic of calcium salts. The crude crystals darkened at temperatures above 300°; a slight residue was left unmelted at 350°. The melting point and solubility data suggested that the dinitrocarbanilide rather than the hydroxy dimethylpyrimidine portion of nicarbazin was the precursor of the isolated material. To obtain purified crystals of the metabolite, a 20 mg sample was dissolved in 2 ml of hot dimethylformamide, centrifuged to separate from foreign material, and then carefully diluted with 1 ml of water. After chilling and collecting the solid, the recrystallization was repeated once more. A third crystallization was then made from about 1 ml of dimethylformamide (without subsequent dilution with water). At this point the material was colorless, darkened above 335°C, and melted with decomposition >345°C. After drying at 100°C for 1 min. the material was subjected to elemental analysis and infra-red study. Qualitative tests for S and halogen were negative while a positive test for N was obtained. Found: C, 62.51, H, 5.45; N, 17.20. The infra-red spectrum (Nujol) contained bands, at 3.0-3.03, 6.0, 6.05, 6.32, 6.48, and 6.57 u. These bands are consistent with NH, amide carbonyl and aromatic residues. A check of the literature indicated that the properties of the isolated compound were in agreement with the previously described 4, 4′-bisacetamidocarbanilide(6). Analysis Calc. $C_{17}H_{18}O_3N_4$ C_1 62.5; H_1 5.56, N_1 17.17.

An authentic sample of the acetamidocarbanilide was prepared by fusion of p-acetamidoaniline and urea(6). No depression of melting point was observed for the mixture of the authentic sample and the isolated metabolite. Also the infra-red spectra of the 2 compounds were identical.

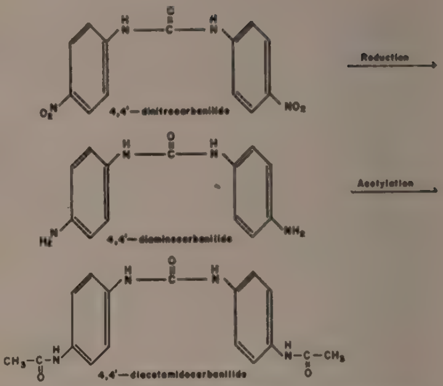

FIG. 2. Metabolic degradation of 4,4′-dinitrocarbanilide in the bovine.

To duplicate the proposed metabolic pathway (Fig. 2), a sample of 4,4′-dinitrocarbanilide was reduced catalytically to the diamine and subsequently acetylated via the Schotten-Baumann procedure. This preparation likewise yielded a compound identical with the metabolite from the kidneys of the calf.

Discussion. In calves, excessively large doses of nicarbazin produced kidney damage and crystalline deposits in the renal tubules. The material has been identified as 4,4′-bisacetamidocarbanilide and it is postulated that the compound was formed by reduction of the aromatic nitro groups of the dinitrocarbanilide component of nicarbazin. The newly formed amino groups were then acetylated into an insoluble metabolite. The pathological lesion and metabolic acetylation of this compound is similar to that resulting from administration of certain sulfonamides (7).

Other studies* have shown that large doses of nicarbazin produced kidney damage associated with crystal deposits in rats but not in dogs. It is well-known that rats possess a marked acetylating capacity whereas dogs are poor in this respect. Therefore, these *in vivo* observations support the suggestion that the metabolic pathway in the formation of 4, 4′-

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* Unpublished data.

bisacetamidocarbanilide involves a reduction of DNC to the soluble diaminocarbanilide, which is readily excreted by dogs but is converted to an insoluble acetylated derivative in rats and calves. The *in vitro* demonstration of the reduction and acetylation of DNC to a compound identical with that found in the kidney tubules further supports the probability of this method for the metabolic degradation of nicarbazin in the bovine.

Summary. The feeding of large doses of nicarbazin to calves produced moderate nephrotoxicity associated with precipitation of crystalline deposits in the renal tubules. These crystals were identified as 4, 4′-bisacetamidocarbanilide. Additional evidence is presented that, after absorption, nicarbazin splits into its components, dinitrocarbanilide and hydroxydimethylpyrimidine, which are metabolized separately. The dinitrocarbanilide is first reduced to soluble diaminocarbanilide and then acetylated to insoluble 4,4′-bisacetamidocarbanilide. The renal tissue reaction and the metabolic degradation of 4,4′-dinitrocarbanilide resembles that known for the sulfonamides.

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1. Cuckler, A. C., Malanga, C. M., Basso, A. J., and O'Neill, R. C., *Science,* 1955, v122, 244.

2. Cuckler, A. C., Malanga, C. M., and Ott, W. H., *Poult. Sci.,* 1956, v35, 98.

3. Porter, C. C., and Gilfillan, J. L., *ibid.,* 1955, v34, 995.

4. Clark, I., Geoffroy, R. F., Gilfillan, J. L., and Porter, C. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 4.

5. Ott, W. H., Kuna, S., Porter, C. C., Cuckler, A. C., and Fogg, D. E., *Poultry Sci.,* 1956.

6. Schiff, H., and Ostrogovich, A., *Ber.,* 1894, v27, 398.

7. Northey, E. H., *The sulfonamides and allied compounds,* Rheinhold Publishing Co., New York, 1948, 451.

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Demonstration of Central Nervous Mediation of Acute Pulmonary Edema Produced by Intravenously Administered Epinephrine. (22722)

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Experiments have been previously reported (1) on the phenomena associated with production of acute pulmonary edema in mice and rats. The most straightforward interpretation of the observations was obtained on the assumption that the genesis of the acute pulmonary edema either produced by air blast or by massive doses of epinephrine was mediated via the central nervous system. As other interpretations were not entirely ruled out, it was considered important to obtain a more rigorous proof of the central nervous mediation, especially when produced by epinephrine. Usually the physiological effects of intravenously administered epinephrine are interpreted in terms of its well-known peripheral effects. However, it can be easily demonstrated that massive and even lethal doses of epinephrine can be administered intravenously to cats without any evidence of acute pulmonary edema production, while the typical acute edema can often be produced by injection of epinephrine into the hypothalamic regions of the brain of the cat. A natural interpretation of these observations is that the cat has a blood-brain barrier more impervious to epinephrine than that of rodents.

To prove conclusively the central nervous mediation of the acute edema in rats, the experiment was performed of severing the spinal cords of a group of rats at a position between the second and third vertebrae. The respiration was maintained with a mechanical respir-

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* This paper is based on work performed under contract No. AT-04-1GEN-12 between Atomic Energy Commission and the University of California at Los Angeles.

TABLE I. Spinal Severance.
Total body weights from 288 to 452 g (avg 351 g)

	Lung wt in g	
	1.923	
	1.227	
	2.295	(respirator blocked)
	1.781	
	2.570	(respirator blocked)
	1.421	
	2.665	(some jerking of hind legs)
	1.705	
	1.312	
	1.417	
	1.166	
	2.683	(some jerking of hind legs)
	1.665	
	2.166	(some jerking of hind legs)
	1.470	
Avg	1.831	

No Spinal Severance (But on Respirator).
Total body weights from 331 to 435 g (avg 383 g)

	4.384	(nasal frothing)
	8.550	
	4.219	
	3.610	(nasal frothing)
	7.063	(*Idem*)
	4.896	(”)
Avg	5.453	

ator. Massive doses of epinephrine, that in controls produced tremendously edematous lungs, were administered intravenously. Under these conditions it would be expected that if the mediation was central nervous, no acute edema would occur in the cord-severed animals, while if the mediation was peripheral, the edema would develop.

Procedure. Rats were anesthetized as required with 0.4 ml to 1.0 ml of a one-gram/10 ml solution of Evipal Sodium administered intraperitoneally. They were laid on their backs and tied to a board by their feet. A tracheal incision was made and a cannula connected with a respirator was inserted and fastened, following well-established methods. The rats were turned over and refastened to the board. An incision was made in the back of the neck and a drill used to sever the cord between the second and third vertebrae. The animal showed a typical reaction to the severance by becoming momentarily rigid and then limp. Rats which withdrew their limbs on pinching were not considered to have their spinal cords completely severed and were not used. The rats were again turned over on their backs and freshly prepared adrenalin, 2 mg/kg, was injected into their femoral veins.

Results. Table I gives the measured lung weights of the experimental animals and a group of controls kept on the respirator and administered the same dose level of adrenalin, but without cord severance. However, incisions were made on the back of the neck of the controls, similar to those on the experimental animals.

These results strikingly demonstrate without the necessity of a statistical analysis that cord severance prevents development of acute edema. The appearance of the lungs in the spinal severance group was not normal. Some had a peculiar spongy appearance but no edema fluid was apparent. The production of simultaneous peripheral effects by the epinephrine is in no way excluded as a possibility. Also, possible mechanical irritation could arise from abnormal inflation during the artificial respiration.

Discussion. Luisada(2) has claimed that pulmonary edema can be mediated via the central nervous system by controlling capillary permeability of the pulmonary capillaries. As there is no anatomical evidence of nervous control of capillary permeability, this hypothesis is not well supported. A hypothesis for a different mechanism of central nervous mediation has been presented by us(1) which seems to fit in well with all the observations to date. In this mechanism only the central nervous control of precapillary sphincters is assumed.

An animal with severed cord on a respirator can be killed with a massive dose of epinephrine, even though no pulmonary edema is generated. It is likely but unproved that the epinephrine produces many other central nervous misfunctions besides that presumably produced on the circulating lung resistance by loss of control of the pulmonary precapillary sphincter system.

Summary. Fifteen rats with severed spinal cords, kept alive on a respirator, were given massive doses of epinephrine, that on controls produced consistently an intense acute pul-

monary edema. None of these rats developed the usual acute edema, and in 10 cases the lungs had normal weight. This result is interpreted as a proof of the central nervous mediation of the acute pulmonary edema produced by administration of epinephrine.

1. Cassen, B., and Kistler, K., *Am. J. Phys.*, July, 1954, v178, 49, 53; *Reports UCLA*-215, 245, 266, and 268.

2. Luisada, *Arch. f. Exp. Path. u. Pharmakol.*, 1928, v132, 313.

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Passive Transfer with Circulating Leukocytes of Delayed Hypersensitivity to Cat Scratch Antigen.* (22723)

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The passive transfer of delayed type skin hypersensitivity in animals and in man has been demonstrated towards simple chemical compounds(1), to tuberculin(2), to whole streptococcal cells, to SK-SD, and to streptococcal M substance(3,4). Such transfer has been possible with the leukocytes from peritoneal exudates, from lymph nodes, from the spleen, and from the thoracic duct, and from certain fractions of disrupted leukocytes(5,6). Hitherto the passive transfer of skin hypersensitivity to virus diseases has not been reported. It is the purpose of this paper to report the successful passive transfer of the delayed type skin hypersensitivity to cat scratch disease by means of circulating leukocytes. Cat scratch disease is a benign, subacute, regional lymphadenitis that is characterized by reticuloendothelial hyperplasia with epithelioid cell granulomas having central necrosis. Patients with cat scratch disease develop a characteristic skin reaction to an antigen prepared from pus aspirated from other cases of cat scratch disease. In its temporal relationships and its morphological features the reaction to cat scratch antigen has features similar to the tuberculin reaction and is characteristic of delayed allergy in man. No causative agent has been isolated from the suppurating lymph nodes from patients with cat scratch disease despite cultures on all commonly used media, upon inoculation into laboratory animals including rabbits, cats, monkeys, guinea pigs, mice, rats, and chick embryos, or on inoculation of tissue culture of HeLa cells, kidney cells, liver cells, or corneal cells.[§] No microorganisms are to be seen in sections of the involved lymph nodes although cytoplasmic basophilic inclusion bodies have been described in the cells of such nodes(7). Further evidence in support of a viral etiology for cat scratch disease is that patients with this disease have a distinctly higher incidence of low complement fixation titers against the lymphogranuloma-psittacosis group viruses than does the population at large(8).

Materials and methods. Obtaining white blood cells. The antecubital area is cleansed with tincture of iodine, alcohol and green soap. The skin is infiltrated with 1% procaine hydrochloride. The antecubital vein is punctured with a 15 gauge needle and the blood is withdrawn through silicone-coated tubing into a silicone coated vacuum bottle containing ACD as anticoagulant. (For volumes of blood less than 400 ml heparin, 5 mg/100 ml blood, is used as the anticoagulant). Twenty-five ml of 4.7% dextran is

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TABLE I.

Donor sensitive to cat scratch disease antigen			Recipient not sensitive to cat scratch disease antigen			No. of viable cells transferred (millions)	Mode of inoculation	Height of reactivity	Duration of reactivity, wk
	Sex	Age		Sex	Age				
C. D.	♀	11	M. M.	♀	3	260	Intramusc. + subcut.	2+	3
P. D.	♀	8	S. M.	♂	5	400	Intramusc. + subcut.	3+	>4
M. D.	♀	46	G. A.	♂	8	1000	Intramusc.	+	2

added for each 100 ml of blood, and the mixture inverted twice. (If heparin is used, normal saline is added in addition to the dextran, 29.5 ml/100 ml of blood). The mixed blood is allowed to stand at room temperature for 50 minutes, while red cells settle at a rate far in excess of the rate for the white cells. The plasma containing the white blood cells is removed with a plasma separating needle and a silicone-coated vacuum flask. The plasma is placed in silicone-coated centrifuge tubes and centrifuged at 1,000 rpm for 20 minutes. The supernatant plasma is poured off and the leukocytes together with about an equal number of admixed red cells are suspended in normal saline. The suspension is taken up into a silicone-coated syringe and injected intramuscularly, or partly intramuscularly and partly subcutaneously. *Handling the white blood cells.* An aliquot of the white cell suspension is used to determine the total and differential counts, and the percentage of viable cells is estimated by vital staining with trypan blue(9). Approximately 200,000,000 white blood cells are obtained/each 100 ml of blood processed, and between 80 and 99% are viable. The cell donors were patients convalescent from cat scratch disease who demonstrated a strongly positive skin test to cat scratch disease antigen. The recipients were children who had negative skin tests for cat scratch disease. Matching for compatibility of red cell groups was carried out because of the admixture of erythrocytes with the leukocytes. The cat scratch antigen was prepared from sterile pus aspirated from 2 carefully characterized cases of cat scratch disease. The pus was diluted 1:5 with pyrogen free normal saline and heated at 56 degrees C for one hour on 2 consecutive days. The resultant antigen in 0.1 ml quantities gave reactions characterized by induration and erythema beginning at 12 hours and lasting for 72 hours to 5-6 days after intradermal injection in subjects with typical cat scratch disease. Reactions on controls were completely negative. In the experimental series, skin tests were applied two days after the transfer of cells, and at weekly intervals thereafter. These tests were read for induration and erythema at 48 hours, as follows: +, 5 to 10 mm; 2+, 10 to 15 mm; 3+, 15 to 20 mm. Ancillary evidence of hypersensitivity was shown by the occurrence of a brownish discoloration after the erythema had faded. This discoloration, which persisted for many weeks in patients with cat scratch disease, lasted as long as the passively transferred reactivity could be demonstrated.

Results. The results summarized in Table I demonstrate the successful passive transfer of cat scratch disease skin hypersensitivity by means of circulating leukocytes in three consecutive cases.

An attempt to transfer the skin reactivity to cat scratch disease by means of plasma from a convalescent patient with a strongly positive skin reaction (4+) was unsuccessful. Four hundred cc of blood were removed from patient M.D. into a standard blood collecting flask with ACD as anticoagulant. After settling of cells the plasma was removed and filtered through a Seitz filter. One hundred and fifty cc of this plasma were given intravenously to recipient M.M. This patient failed to develop positive skin reactivity to cat scratch disease antigen.

Summary. Delayed type hypersensitivity to cat scratch disease can be passively transferred by blood leukocytes, but not by plasma from hypersensitive donors convalescent from the disease.

1. Eisen, H. N., and Belman, S., *J. Exp. Med.*, 1953, v98, 523.

2. Chase, M. W., PROC. SOC. EXP. BIOL. AND MED., 1945, v59, 134.

3. Lawrence, H. S., *J. Immunol.*, 1952, v68, 159.

4. ———, *Streptococcal Infections,* Edited by McCarty, M., New York, 1954, Columbia University Press.

5. ———, *J. Clin. Invest.*, 1955, v34, 219.

6. ———, *Am. J. Med.*, 1956, v20, 428.

7. Mollaret, P., Reilly, J., Bastin, R., and Tournier, P., *Presse Med.*, 1951, v59, 701.

8. ———, *ibid.*, 1951, v59, 681.

9. Harris, S., Harris, T. N., and Farber, M. B, *J. Immunol.*, 1954, v72, 148.

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Effects of Azetazoleamide (Diamox) Upon Blood Sugar of Normal and Diabetic Patients. (22724)

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Moseley *et al.*(1) observed insulin shock in a diabetic patient receiving azetazoleamide as a diuretic and subsequently made studies on normal subjects to evaluate the effect of this drug on glucose tolerance. No significant change in the intravenous glucose tolerance curve was noted when studied with and without the administration of azetazoleamide. They concluded from the oral glucose tolerance curve that azetazoleamide may interfere with the absorption of glucose from the gastrointestinal tract.

Since Moseley did not study diabetic patients and limited his study to an acute problem, our studies were designed to determine whether azetazoleamide influences the fasting blood sugar of normal and diabetic patients after acute and chronic administration and whether the oral glucose tolerance curves can be affected.

Acute study. Acute experiments were carried out in 20 normal and 20 diabetic subjects. Half of each group were given either 500 mg of azetazoleamide* orally and fasting blood sugars determined (method of Folin-Wu)(2) 3 hours later or followed without drug administration for a similar period. One week later, to eliminate biologic variation, the subjects were re-studied but the procedure reversed. The results of this experiment are summarized in Table I. Azetazoleamide did not alter the fasting blood sugar 3 hours after its administration in the normal and the diabetic group of patients when studied as an acute experiment. The blood sugar taken at 3 hour intervals was practically the same with and without the drug.

Chronic study. Chronic experiments were carried out in 6 normal and 6 diabetic patients. Azetazoleamide was given in doses of 500 mg daily for a 3 week period to 3 normal and 3 diabetic patients. Fasting blood sugars were determined (method of Somogyi)(3) at weekly intervals. As a control, a similar group was followed without drug administration. In Table II average values of blood sugars are recorded. With chronic administration of 500 mg of azetazoleamide there was no significant change in the fasting blood sugar in either the control patients or those receiving the drug for a period of 3 weeks.

Oral glucose tolerance. The final experiment was related to the effect of azetazoleamide on the glucose tolerance curve. Three normal and 3 diabetic patients received the standard oral glucose tolerance test utilizing 100 g of glucose. The procedure was repeated at a later date with 500 mg of azetazoleamide being given 3 hours prior to the

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* Supplied by Lederle Laboratories Division, American Cyanamid Co.

TABLE I. Influence of Azetazoleamide upon Blood Sugar of Normal and Diabetic Patients. (Value of fasting blood sugar in mg %.)

	Without drug				After oral admin. of 500 mg			
	Control		3 hr		Control		3 hr	
Subjects	Range	Avg	Range	Avg	Range	Avg	Range	Avg
Non-diabetic (20)	95-132	114	100-124	112	83-140	112	93-132	113
Diabetic (20)	85-380	233	83-320	202	106-367	237	103-340	222

TABLE II. Chronic Experiment on Normal and Diabetic Patients. (Avg value of fasting blood sugar in mg %.)

	Control*				500 mg azetazoleamide O.D. daily*			
Weeks	0	1	2	3	0	1	2	3
Non-diabetic	86	84	91	97	79	76	82	87
Diabetic	137	135	144	158	118	118	120	127

* 3 subjects for each group.

administration of glucose. The glucose tolerance curves are recorded in Table III. Although there was considerable individual variation in the curves obtained, the blood sugar curve for each of the subjects studied was not different with and without azetazoleamide. The differences in the curves obtained are within the range of technical and biologic variation.

TABLE III. Effect of Azetazoleamide on the Glucose Tolerance Curve. (100 g glucose orally—value of fasting blood sugar in mg %.)

		Control				500 mg azetazoleamide 3 hr before test			
	Min.	0	30	90	150	0	30	90	150
Non-diabetics	1. A.B.	76	109	107	100	79	106	106	101
	2. C.P.	88	138	126	115	90	123	104	101
	3. J.K.	89	162	200	138	100	151	132	112
Diabetics	1. H.D.	127	200	308	313	128	181	268	258
	2. A.F.	96	99	172	236	107	112	189	245
	3. H.B.	159	192	267	300	178	213	251	305

Comment. Maren *et al.*(4) previously observed in chronic acidotic dogs maintained on azetazoleamide that the fasting blood sugar had slight but definite tendency to rise. However, an insulin tolerance test was found to be normal in a dog treated with azetazoleamide.

The response that Moseley noted was probably due to a well recognized clinical observation of diabetic patients with congestive heart failure. During this time they may be uncontrollable and require an increased dose of insulin. As the congestive heart failure improves, the insulin requirements may be decreased and eventually discontinued. Since azetazoleamide is used in diabetic patients for its diuretic properties, it is conceivable that the drug resulted in a change of cardiac status and insulin requirements, and azetazoleamide *per se* has no significant effect on sugar metabolism or insulin sensitivity. Any diuretic agent would have the same action and it is unnecessary to postulate a specific effect of azetazoleamide upon the blood sugar of diabetic subjects.

Conclusions. 1. The influence of azetazoleamide upon fasting blood sugar and the glucose tolerance curve was studied in a group of diabetic and normal subjects. Azetazoleamide was given either as a single dose or for a period of 3 weeks. 2. No significant alteration of the fasting blood sugar or the glucose tolerance curve was noted following the administration of azetazoleamide.

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1. Moseley, V., McCord, W. M., Baroody, N. B., Weeks, W. H., and Ivester, M. E., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 498.

2. Folin, O., and Wu, H., *A System of Blood Analysis, J. Biol. Chem.*, 1920, v41, 367.

3. Somogyi, M., *ibid.*, 1945, v160, 61.

4. Maren, T. H., Mayer, E., and Wadsworth, B. C., *Bull. Johns Hopkins Hosp.*, 1954, v95, 199.

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Failure to Demonstrate Capsular Swelling in *Cryptococcus neoformans*.* (22725)

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The capsule which surrounds certain microorganisms is ordinarily water-clear and difficult to see without appropriate staining procedures. If, however, specific antibody is added, a reaction occurs which renders the capsule clearly visible. This antigen-antibody reaction which was first demonstrated with the pneumococcus(1) has been of interest not only for identifying and classifying microorganisms, but also because it affords direct cytological evidence for the combination of antibody with a surface antigen. Although this combination is frequently referred to as the "Quellung" or "capsular swelling" reaction, several investigators(2-5) have recently suggested that the capsule does not actually increase in size in bacteria like pneumococcus, the *Klebsiella* group and the *Bacillus* group. Tomcsik in discussing the morphological arrangement of immunopolysaccharides in the bacterial cell(5) suggested that the essential feature of the reaction is precipitation and that swelling might be a secondary phenomenon due probably to capsular hydration. Johnson and Dennison(6) presented experimental data which indicated that a volume increase actually occurs when the pneumococcus capsule reacts with antibody. This is in conflict with the results of the previously mentioned authors as well as with work on the capsular reaction of *Cryptococcus neoformans* by Neill, *et al.*(7) and by members of our group(8).

The purpose of the present study is to describe further experiments on this interesting reaction. *Cryptococcus neoformans* has been used as the test organism since its relatively large size and spherical shape facilitate direct microscopic measurements.

Methods. Two strains of *C. neoformans* were selected because of their ability to produce relatively large capsules. Both strain DU (Type A) and strain L2 (Type B) were isolated from patients at the Los Angeles County General Hospital. Cultures were maintained on neopeptone-glucose medium (8). In certain experiments, cells with larger capsules were obtained by injecting mice intraperitoneally with 4 x 10^9 *C. neoformans* and washing the cells from the peritoneal cavity with saline solution on the sixth day of infection. Phenol, 0.5%, was added to cell suspensions that were to be stored for a period of time. All cells were centrifuged and washed 4 times with saline solution immediately before use to remove dissolved polysaccharide. Antiserum was prepared in rabbits as described in an earlier report(9). Antiserum No. 12 was polyvalent with respect to Types A, B, and C. Serum taken from rabbits before immunization was used for control experiments. Among the methods used to study possible change in capsular size during the antigen-antibody reaction was the measurement of packed volumes of centrifuged cells with and without added antibody. For these experiments, strain DU was grown in neopeptone-glucose broth for 4 days at 25-27°C with continual agitation, and then killed by the addition of phenol to a final concentration of 1%. Suspensions of strain L2 from infected mice were also used. Cells were removed by centrifugation, washed four times with saline solution and resuspended in saline or phosphate buffer solutions at pH 7.0 ($\mu = 0.1$). The cell concentration was adjusted to 85% transmittance in a Klett Summerson

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TABLE I. Volumes of Packed Cells following Two Hours Centrifugation at 2500 × Gravity.

Diluent	Cell strain	Serum dilution	Anticryptococcal serum #12 (mm^3)	Normal rabbit serum (mm^3)
Phosphate buffer pH 7 ($\mu = 0.1$)	DU	1: 4	12	13
		1: 16	12	13
Saline solution	"	1: 4	13	14
		1: 16	13	13
Saline solution	L2	1: 8	30	37
		1:160	30	36

DU strain was harvested from cultures and the L2 strains from infected mice. Capsules of the latter strain were extremely large (see Table III for mean diameters).

Colorimeter using a No. 54 filter. Both normal rabbit serum and anticryptococcal serum for use in the experiment were subjected to preliminary centrifugation at 2500 x g for 30 minutes although both had been clarified by filtration. To Hopkins centrifuge tubes were added 4 ml of cell suspension and 4 ml of a given serum dilution. Following a 5 minute incubation at room temperature, a sample was taken for microscopic examination to verify the presence or absence of a capsular reaction. The tubes were then centrifuged at 2500 x g for 30 minutes and the cell volume noted. The tubes were centrifuged at the same force for additional 30 minute periods until a total time of 2 hours centrifugation was reached. Similar experiments were conducted at 400 x g and 1700 x g and with saline or cultural supernate replacing the phosphate buffer as diluent. Although other investigators have found forces above 2500 x g necessary for demonstrating volume changes in pneumococcus(6), increasing the force above this level did not alter the relative volume in experiments with cryptococcus. For direct microscopic measurement of capsular diameter, suspensions containing 10^7 cells per ml of saline solution were used. In one procedure, individual cells were located on a slide containing no serum and just enough India ink to permit the capsular outline to be seen. The diameters of the capsule and of the somatic portion of the cell were then measured with a Carl Zeiss filar micrometer. Following this, diluted antiserum was allowed to flow under the cover glass while the cell was held in the microscopic field. The same cell was measured again after it had produced a capsular reaction. In other experiments, 0.2 ml portions of cell suspension were placed in 10 x 75 mm test tubes. To some tubes were added 0.04 ml of antibody solution, to others, 0.04 ml of dilute India ink. After mixing, a drop of each was added to cover glasses for the preparation of hanging drop or flat slides. In other experiments, a counting chamber of uniform (0.1 mm) depth was used. In some instances, India ink was added to slides containing cells plus anticryptococcal serum and to control slides with cells and normal serum. After an incubation period of 5 to 15 minutes at room temperature, the diameters of 30 cells were measured with an ocular micrometer. The averages of these values were then calculated.

Results. The results of centrifuging Cryptococcus (DU) cells for 2 hours at 2500 x g are presented in Table I. There was no significant difference in packed volumes of aliquots of cells which had been mixed with specific antiserum and with normal rabbit serum. The substitution of saline for phosphate buffer had no effect on the results. Decreasing the force to 1700 or 400 x g failed to disclose any volume increase in the cells mixed with antiserum. In some experiments, the volume was slightly greater in normal serum which could have been due to shrinkage or to firmer packing in the antigen-antibody aggregate. When the experiment was repeated with cells possessing extremely large capsules (diameter 28 to 50 μ), there was evidence of capsular shrinkage in the presence of antibody. Dilution of the antibody to the point where the capsular reaction was relatively weak (one twofold dilution be-

TABLE II. Effect of Dissolved Polysaccharide in Cultural Supernate on Apparent Packed Cell Volume.

Diluent added to washed cells	Serum (1:8) Anticryptococcal #12 (mm³)	Normal rabbit serum (mm³)
Saline	10	10
Cultural supernate		
1: 4	>50*	11
1:20	39	10

* The packed cell-precipitate mass greatly exceeded the graduations in the tube. The vol was estimated to lie between 300-400 mm³.

Tubes were centrifuged at 2500 × gravity for 2 hr.

Strain DU was used in this exp.

low the extinction point) did not change the relative difference between tubes with antiserum and those with normal serum.

The effect of extracapsular polysaccharide on the apparent volume of packed cells is shown in Table II. It may be seen that when washed cells were resuspended in the culture fluid in which they had been grown and then mixed with antiserum, a great increase in volume resulted as compared to values in normal serum. This represents precipitation of dissolved polysaccharide and possibly other antigens present in the supernatant cultural fluid.

Direct microscopic measurements on single cells before and after the addition of antibody demonstrated that there was no swelling and indeed, the capsules appeared to shrink when antibody was allowed to flow under the cover glass. Control slides in which additional India ink was substituted for antibody also showed some degree of shrinkage, indicating that at least part of the observed decrease in diameter was due to mechanical lifting of the cover glass, permitting the flattened capsule to assume a spherical shape. Because of the technical shortcomings of this method, another procedure was adopted. Two hanging drop slides were prepared, one with cell suspension plus antiserum, the other with cell suspension plus India ink. The diameters of 30 cells were measured on each slide, and the mean diameter calculated. Similar experiments were set up using a counting chamber of uniform depth and a flat microscope slide with a cover glass. In no case was capsular swelling observed (Table III); moreover, mean capsular diameters of the cells in antiserum were significantly lower than those in India ink.

Discussion. In studying the mechanism of this antigen-antibody reaction, it is important to know whether swelling of the capsular polysaccharide-antibody complex is an essential feature of the reaction. The data presented here indicate that it is not. The difference in our results and those of others(6) cannot be adequately explained without further work. A different organism, the pneumococcus, was used in the earlier study and it is possible that the capsular reaction differs among different species or types depending on the physicochemical nature of the capsular antigen.

The pneumococcus is known to have an extra-capsular slime layer(2,10) and this may be responsible for the apparent swelling in the pneumococcal capsule. It may be significant that previous investigators(6) did not wash their cells to free them of extra-capsular polysaccharide. In their centrifugation experiments, the apparent increase in volume may have been due to precipitate. In our experiments with cryptococcus, a 1:4 dilution of cultural supernate added to washed crypto-

TABLE III. Comparison of Means of Capsular Diameters in India Ink and in Antiserum.

Exp. No.	Treatment	Mean diameter (μ)	S.D.	S.E.	P value
1	India ink	36.72	2.18	.40	<0.001
	Antiserum	34.62	2.00	.37	
2	India ink	38.43	3.22	.59	"
	Antiserum	34.90	2.66	.49	
3	India ink	38.00	3.43	.63	"
	Antiserum	34.76	2.49	.46	
4	India ink	43.86	2.28	.42	"
	Antiserum	41.16	2.21	.40	

Means were calculated from measurements on 30 cells each. Exp. 1 was done with a hanging drop slide; Exp. 2 and 3 in a counting chamber; Exp. 4 on a flat slide with a cover glass.

Abbreviations: S.D., stand. dev.; S.E., stand. dev. of the mean; P, probability calculated by Student's "t" test.

Strain L2 harvested from mice was used for these experiments.

coccal cells yielded so much precipitate that the total mass remained far above the graduated level in Hopkins tubes. The difference between this and the normal serum control was indeed striking, and could have been interpreted as capsular swelling had not the experiment with washed cells been conducted.

Although there is no capsular swelling, the data presented here suggest that there is some degree of capsular shrinkage. This is particularly apparent in cells harvested from mice because these cells have extremely large capsules. Further experimentation will be necessary to determine whether this apparent shrinkage has biological significance although from a statistical standpoint the differences in mean diameters presented in Table III are significant. It is possible that the apparent shrinkage seen under the microscope does not represent any volume change in the capsule but merely the change from an ellipsoid to a sphere during the antigen-antibody reaction. A shortcoming of the direct microscopic measurement is the fact that capsular boundaries in India ink are not as sharp as in antiserum, particularly in the counting chamber where the fluid depth is 0.1 mm. This accounts for the somewhat larger standard deviations obtained in counting chamber experiments. This criticism cannot be directed at centrifugation experiments; however, it is possible that the shrinkage observed here represents better packing in the presence of antibody.

Summary. 1. In studying the mechanism of the "capsular reaction" one of the first questions to be answered is whether or not the capsule swells when antibody is added. Data presented here indicate that it does not, using *C. neoformans* as a test organism. Exposure of washed, encapsulated *C. neoformans* to antibody results in a prominent clarification of capsular outline, but no increase in capsular size as determined by packed cell volume after centrifugation and by direct microscopic measurement. When cells with large capsules are employed, there is an apparent *decrease* in capsular size during the reaction with antibody. 2. If capsulated cells are not washed free of soluble capsular polysaccharide present in the cultural supernate, the soluble antigen coprecipitates when antibody is added. This may lead to the erroneous conclusion that there is an increase in capsular volume.

1. Neufeld, F., *Z. Hyg.*, 1902, v40, 54; Neufeld, F., and Etinger-Tulczynska, *ibid.*, 1931, v112, 492.

2. Klienberger, Nobel E., *J. Hyg.*, 1948, v46, 345.

3. Duguid, J. P., *J. Path. and Bact.*, 1951, v63, 673.

4. Edwards, P. R., and Fife, M. A., *J. Inf. Dis.*, 1952, v91, 92.

5. Tomcsik, J., *Ann. Rev. Biochem.*, 1953, v22, 351.

6. Johnson, F. H., and Dennison, W. L., *J. Immunol.*, 1944, v48, 317.

7. Neill, J. M., Castillo, C. G., Smith, R. H., and Kapros, C. E., *J. Exp. Med.*, 1949, v89, 93.

8. Evans, E. D., *J. Immunol.*, 1950, v64, 423; Banish, G., and Evans, E. E., unpublished data.

9. Evans, E. E., Sorensen, L. J., and Walls, K. W., *J. Bact.*, 1953, v66, 287.

10. Wood, W. B., and Smith, M. R., *J. Exp. Med.*, 1949, v90, 85; ———, *ibid.*, 1950, v92, 85.

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Effect of Biotin Deficiency on Follicular Melanocytes of Mice. (22726)

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It has been known for some time that pigmented mice and rats produce grey hairs when made biotin deficient(1,2,3). Unlike greying induced by X-rays, which is permanent(4), greying resulting from biotin deficiency is reversible(3). It would appear that melanocytes persist within the hair follicles of biotin deficient mice whereas they are destroyed in X-irradiated mice. Thus far, no attempt has been made either to trace the fate of melanocytes within the greyed hair follicles of biotin deficient mice or to determine the extent to which such depigmented melanocytes are morphologically and physiologically similar to the "pigmentless" melanocytes (clear cells) of albino mice. In addition, whereas it is well established in mice that the synthesis of the black-brown melanins (eumelanins) is inhibited by biotin deficiency, it is not known whether yellow melanin (phaeomelanin) synthesis is similarly affected.

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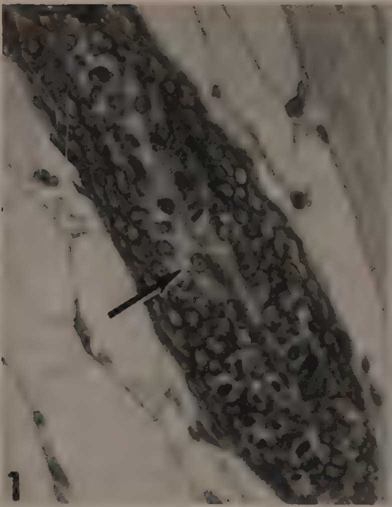
It is the purpose of this paper to describe the effects of biotin deficiency on the follicular melanocytes of both yellow (A^y) and black (C57BL) mice.

Materials and methods. To produce biotin deficiency, 25 black (C57BL) and 5 yellow mice, 16 days of age, were weaned to diet #9A described by Wilson *et al.*(2). The effectiveness of this diet in causing greying of hair in C57Black mice was demonstrated by these investigators and subsequently by Rauch(3). Biopsies of skin were taken approximately 20 days post-weaning, shortly after the hairs of the second coat grew visibly above the skin. The skin samples were placed in Bouin's fixative and routine histological procedures were followed. Paraffin sections were cut at 7 μ and stained with Delafield's hematoxylin. Sections of depigmented follicles of the biotin deficient mice were compared with the follicles of albino mice in a comparable stage of hair growth. The activity of the dopa oxidase enzyme system within the depigmented melanocytes was determined by testing their response to exogenously supplied d,l-3-4-dihydroxyphenylalanine (dopa). Fresh frozen sections of skin from biotin deficient C57Black mice were cut on the freezing microtome at 30 μ and incubated in a buffered 0.76M solution of dopa according to the method of Laidlaw and Blackberg(5). Control sections were incubated in buffer alone. In addition, fresh frozen sections of skin from normal C57Black mice were treated in identical fashion, some sections being incubated in dopa solution, others in buffer alone.

Results. The pattern of greying is the same in C57Black mice and in yellow mice weaned to the experimental diet. The first hair growth cycle is completed approximately 2 days post-weaning when the mice are 18-19 days old. The hairs of the first generation are normal in pigmentation. The hairs of the second generation, appearing above the skin at approximately 20 days post-weaning, show a marked reduction in pigment content. Longitudinal sections of the hair follicles reveal a similar reduction in the amount of pigment within the hair bulbs. Except for the presence of small granules of pigment within the melanocytes and recipient epithelial cells, the greyed hair follicles are strikingly similar to those of the albino (Figs. 1, 2). The depigmented melanocytes contain a large hyalinated cytoplasm as do the clear cells or "pigmentless" melanocytes of the albino. Depigmented melanocytes are found in the hair bulbs of both biotin deficient black and yellow mice and in all instances they are restricted to the upper halves of the hair bulbs. This is also the position of clear cells within the hair bulbs of the albino mouse.

Depigmented melanocytes resulting from biotin deficiency react positively to exogenously supplied dopa. Abundant dopa melanin is observed in the hair follicles which are incubated in dopa solution (Fig. 3). The amount of dopa melanin formed indicates clearly that with regard to the activity of the dopa oxidase system, the melanocytes are capable of greater melanin synthesis than is expressed in the biotin deficient individual. In this way depigmented melanocytes differ from clear cells which have been shown to be dopa negative(6).

Discussion. The present experiments demonstrate that melanocytes persist within the depigmented hair follicles of biotin deficient pigmented mice and that they are similar in morphology to the follicular clear cells of albino mice. This observation lends support to the view held by previous investigators (7,8) that the clear cells of the albino are melanocytes which are incapable of melanin synthesis due to a gene controlled enzymatic deficiency. Unlike clear cells, however, de-



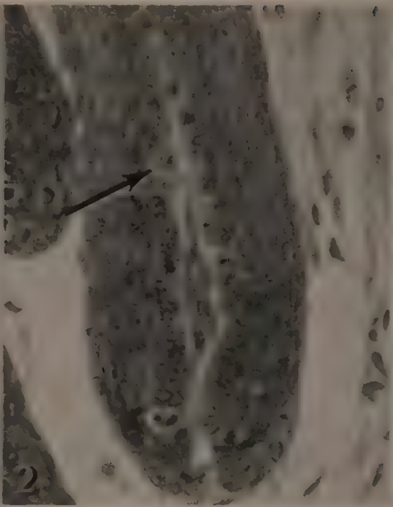


FIG. 1. Vertical section through a hair follicle of the albino mouse. Note the large clear cells (arrow) in the upper bulb. ×430.

FIG. 2. Longitudinal section of a hair follicle from a biotin deficient C57 Black mouse. Depigmented melanocytes (arrow) are visible in the upper bulb. ×430.

pigmented melanocytes retain the ability to respond *in vitro* to dopa. It is probable that the histological appearance of greyed hair follicles observed in biotin deficiency is also typical for the reversibly greyed hair follicles resulting from other nutritional deficiencies, *e.g.*, copper, zinc, and pantothenic acid deficiency(9). However, it would not be surprising if the response of depigmented melanocytes to exogenous dopa is subsequently found to vary considerably with the type of nutritional deficiency.

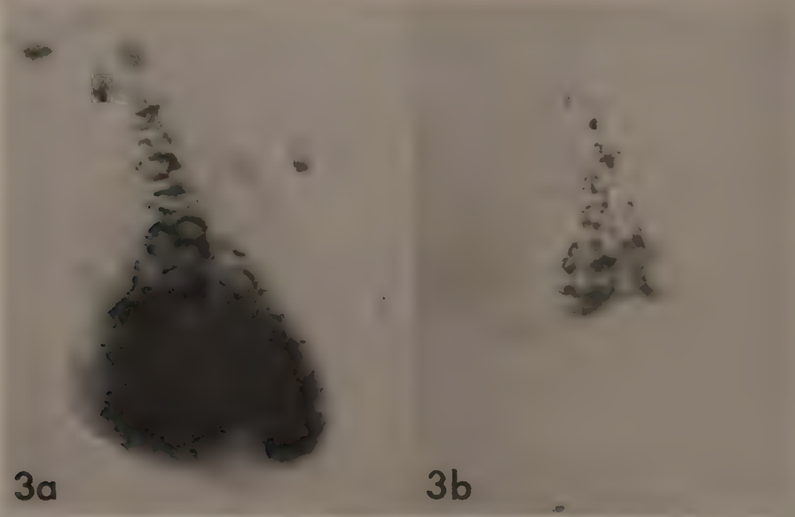


FIG. 3. a. Frozen section of a hair follicle from a biotin deficient C57 Black mouse, following incubation of the section in dopa. ×430. b. Control section to that shown in a., incubated in phosphate buffer only. ×430.

It would appear that both phaeomelanin and eumelanin synthesis are equally sensitive to biotin deficiency. In both yellow mice and black mice deficient in biotin a marked reduction in pigmentation is found in the hairs of the second hair generation. At present, with the role of biotin in the pigmentary process undetermined, no statement can be made as to the nature of this inhibition.

Summary. Both yellow (A^y) and black (C57BL) mice produce grey hairs when weaned to a diet known to cause biotin deficiency. Histologically, the greyed hair follicles of biotin deficient mice bear a striking resemblance to the hair follicles of albino mice. The depigmented melanocytes of biotin deficient mice are similar in morphology to the follicular clear cells of the albino. Unlike clear cells, however, depigmented melanocytes react positively to tests for the presence of dopa oxidase.

1. Emerson, G. A., and Keresztesy, J. C., PROC. SOC. EXP. BIOL. AND MED., 1942, v51, 358.
2. Wilson, J. W., Leduc, E. H., and Winston, D. H., *J. Nutrition,* 1949, v38, 73.
3. Rauch, H., *Physiol. Zool.,* 1952, v25, 145.
4. Chase, H. B., *J. Morphol.,* 1949, v84, 57.
5. Laidlaw, G. F., and Blackberg, S. N., *Am. J. Path.,* 1932, v8, 491.
6. Russell, L. B., and Russell, W. L., *Genetics,* 1948, v33, 237.
7. Taylor, A. C., *J. Exp. Zool.,* 1949, v110, 77.
8. Silvers, W. K., Thesis, University of Chicago, 1954.
9. Frost, D. V., *Physiol. Revs.,* 1948, v28, 362.

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Measurement of Total Body Water in Sheep Using I^{131} Labeled 4-Iodo-Antipyrine. (22727)

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Antipyrine has been employed extensively as an indicator for the *in vivo* estimation of total body water in man(1,2) and animals (3-6). The method is based upon the dilution principle and is an adaptable and economical procedure for studies with the intact animals. Despite certain theoretical shortcomings(7,8) this method has been found to compare favorably with parallel procedures for body water measurement employing whole body analysis(3-5), specific gravity(6,9,10, 11) or the isotope dilution technic using deuterium(7), or tritium oxide(12). However, these methods require tedious procedures of sample preparation and time consuming analyses that discourage their use in routine experimentation. The recent introduction of I^{131}-labeled 4-iodo-antipyrine as a simple and rapid method for estimating total body water in man(13) made feasible the adaptation of this procedure for use with farm animals(14). This study is concerned with comparative behavior and volume of distribution of antipyrine and I^{131} labeled 4-iodoantipyrine for total body water estimation in sheep.

Materials and methods. The animals used in these studies were normal, healthy individual wether lambs and mature sheep maintained under typical summer and fall farm management conditions. The procedures employed for handling animals and performing quantitative injections of test substances have been described(15). Animals were taken off

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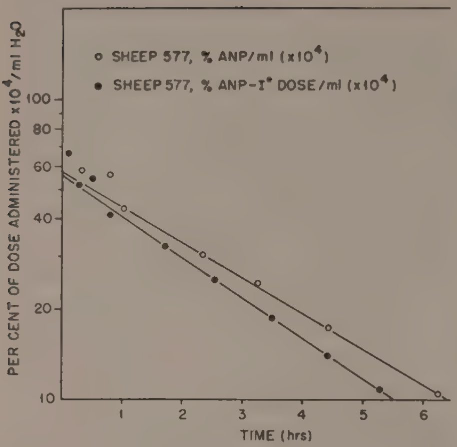

FIG. 1. Log concentration of antipyrine and I^{131} 4-iodo-antipyrine in blood of an avg sheep (577) regressed on time in hr after inj. by method of least squares to derive the theoretical concentration of total body water.

feed and water 12 to 15 hours prior to dosing intravenously with approximately 2 grams antipyrine and/or 50 microcuries I^{131} labeled 4-iodo-antipyrine/100 lb body weight. Venous blood and urine samples were taken periodically during the first 5 hours and at 24 hours after injection. The blood was centrifuged and plasma removed. Radioactivity was measured in four ml of plasma using a well-type sodium iodide scintillation detector attached to a conventional type scaler unit. Radioactivity in an aliquot of standard solution was measured in the same way for calculation of relative iodine content. Total antipyrine concentration was determined by precipitation procedure(16). Radioactivity in both plasma and zinc filtrate fraction was measured for comparative purposes, and for estimation of plasma protein binding. The volume of distribution for each test substance was then calculated by plotting concentration against time after injection on semi-logarithmic paper and exterpolating back to zero time by the method of least squares. (Fig. 1). The zero value was corrected for plasma water by dividing by 0.93, and the test substance injected divided by the concentration in plasma water to give total volume of body water. Body water in liters divided by body weight (kg) X 100 gave the % body water. Thyroid uptake of I^{131} was checked periodically and at 24 hours after administration of labeled 4-iodo-antipyrine using an adapted scintillation probe attached to a count-rate meter.

Results. The adaptation of a new test substance requires that certain physiological studies be made for comparison with proven and acceptable methods of analysis. Since the antipyrine procedure has been used generally for estimation of body water volume in man and animals and has compared favorably with various other procedures(4,5,11, 13), it was of interest to consider results from iodinated antipyrine with data obtained concurrently with stable antipyrine. The use of the labeled compound further permitted a simple investigation of rate and behavior patterns of test substances.

Partition in the plasma. The uniform distribution of both antipyrine and the I^{131} labeled 4-iodo-antipyrine in sheep occurs in about one hour (Fig. 1). The initial rate of disappearance for the 2 test substances was not much different, averaging approximately 30% per hour for animals in the 2 age groups. If it is assumed that the labeled and carrier antipyrine are similarly metabolized by sheep, then the degree of antipyrine binding by plasma proteins may be determined simply by measuring plasma radioactivity before and after deproteinization with zinc hydroxide (1). Furthermore, if plasma could be used directly without deproteinization to reflect the water content from radiochemical analysis, the procedure would be simplified. Therefore, all blood samples were centrifuged and aliquots measured for radioactivity before and after deproteinization and the % reversibly bound to plasma(1) proteins calculated

$$\text{as } 100 \times \frac{\%\text{ dose plasma } I^{131}/\text{cc minus }\%\text{ dose filtrate } I^{131}/\text{cc}}{\%\text{ plasma } I^{131}/\text{cc}}.$$

Results from analysis of plasma samples taken periodically from sixty-two sheep indicated that 10 to 20% of plasma iodine was bound to proteins during the critical 5 hour test period and increased to 60% of that remaining in the blood at 24 hours. In quantitative studies where *absolute* volume data are

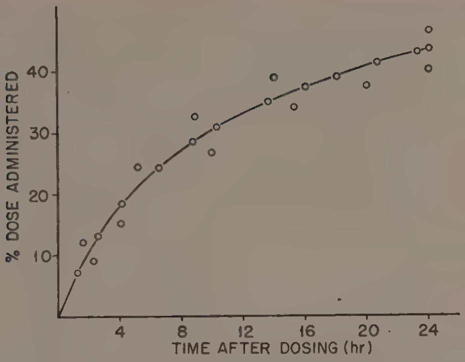

FIG. 2. Accumulative percent excretion of I^{131} in urine of sheep dosed intrav. with I^{131} labeled 4-iodo-antipyrine.

required corrections, therefore, would be necessary. However, in those studies where relative body water volume values are desired, the simplicity of the plasma analysis procedure would be justified.

Thyroid uptake. Measurements of thyroid uptake of I^{131} from labeled antipyrine were made periodically throughout the test period by external counting over the thyroid area with an adapted probe type scintillation counter. The fact that only 3 to 5% of the dose administered was found in this area during the 5 hour test period would indicate the degree of firmness of I^{131} binding to the antipyrine. Six wethers were sacrificed after 24 hours and the thyroids analyzed radiochemically were found to contain 35 ± 8 percent of the I^{131} administered.

Excretion of metabolized antipyrine. Urinary and fecal excretion values and radioanalysis of gastrointestinal tract contents from 8 sheep sacrificed 24 hours after administration of test substances indicated that insignificant amounts of I^{131} were excreted in feces. The accumulative average % of administered dose excreted in urine of 12 sheep is shown in Fig. 2. Of 44% voided during the first day, approximately 20% was excreted during the critical 5 hour test period of the study.

Total body water in sheep. The results of total body water determinations using both antipyrine and I^{131} labeled 4-iodo-antipyrine procedures with 62 young and mature sheep are shown in Table I. These values for young lambs are slightly higher than the 59 ± 5.1 and 61 ± 3.4% reported earlier(14) using antipyrine and I^{131} labeled 4-iodo-antipyrine concurrently in 36 trials with 12 growing lambs. This is probably due to the greater number of younger lambs used in the current study since the young animals have invariably demonstrated a higher body water volume. Mature wethers showed a lower body water volume and checked closely with reports for beef cattle(3), averaging 55.3 ± 6.3 and 56.2 ± 6.6% body water respectively by the antipyrine and labeled antipyrine procedure. The range shown for the two methods was not much different, and reflected the rate of disappearance from plasma (Fig. 1), indicating results from the isotope procedure to compare favorably with that for the stable antipyrine method. The advantage of the labeled antipyrine method lies in simplicity and possible rapidity. It is necessary only to inject intravenously and quantitatively the activity in a minimum volume and to measure the radioactivity in the plasma of blood samples drawn periodically over a 1 to 5 hour period (13). The fact that the labeled test substance appeared to give a slightly higher body water volume may well be accredited to inherent errors involved in quantitative admin-

TABLE I. Percent of Body Water in Young and Mature Sheep as Measured by Antipyrine and I^{131} Labeled 4-Iodo-Antipyrine.

Age	Wt, lb	No. of animals	Method*	Mean†	Stand. dev.	Range
Young	65 ± 10	34	A.N.P.	61.8	6.5	45-72
	62 ± 12	44	I^{131} A.N.P.	62.3	6.8	48-74
Mature	135 ± 49	16	A.N.P.	55.3	6.3	37-63
	138 ± 51	18	I^{131} A.N.P.	56.2	6.6	35-65

* Refers to antipyrine (A.N.P.) and I^{131} labeled 4-iodo-antipyrine (I^{131} A.N.P.).

† Percent body water calculated as $100 \times \frac{\text{Body water (l)}}{\text{Body wt (kg)}}$.

istration of very small volumes of solution. The activity usually required was contained in 0.25 to 1.0 ml. It should be noted, however, that for dilution methods in general the experimental errors almost invariably lead to high values for body water. Some of the more important sources of error include (1) nonquantitative injection into the blood stream, (2) excess water in the gastrointestinal tract and, (3) rapid metabolism of the test substance or its removal from the circulating blood following administration. In this study every precaution was taken to eliminate or to correct for these errors, and by management practices to avoid physiological changes that are known to affect the normal individual.

Discussion. The ultimate objective of establishing a predictable relationship between body water and weight in farm animals is obviously that of using such data to evaluate gain or loss of body energy in nutritional investigations or physiological studies without the need for sacrificing the animal. Several investigators have demonstrated the relative merits of estimating body fat from body water measurements(3,4,5,11,14) and mathematical expressions have been proposed that would permit estimations for other body constituents(17). It is evidenced from the data presented that, although the I^{131} labeled 4-iodo-antipyrine procedure does not rule out all of the recognized shortcomings of the antipyrine method, it offers a simple and rapid means of estimating *relative* body water in mature and in growing farm animals, and is well adapted for routine application to experiments requiring a measurement of this vital metabolic pool. These values employed, for the calculation of total body fat and their extension to prediction of chemical composition and energy values(17) would thereby provide additional criteria for the response measurement of animals to various experimental treatments.

Summary. A simple and rapid procedure is presented for the *in vivo* measurement of total body water in sheep using I^{131} 4-iodo-antipyrine as the test substance. Body water values for 62 young and mature sheep averaged 61.3 ± 6.5 and 55.3 ± 6.3, respectively, by the antipyrine method, and 62.3 ± 6.8 and 56.2 ± 6.6%, respectively by the labeled antipyrine procedure. Thyroid uptake of I^{131} from labeled test substance amounted to 3 to 5% and urinary excretion was less than 20% of administered dose during the critical 5-hour test period. Protein binding was evidenced, indicating that in sheep both test substances were reversibly bound to plasma protein. Consideration is given to the use of these body water volume values in establishing a predictable relationship to body constituents and to provide additional criteria for estimating animal response to experimental treatments.

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1. Soberman, R., Brodie, B. B., Levy, J. B., Axelrod, J., Hollander, V., and Steele, J. M., *J. Biol. Chem.*, 1949, v179, 31.

2. Hamwi, G. J., and Urbach, S., *Metabolism*, 1953, v2, 391.

3. Kraybill, H. F., Hankins, O. G., and Bitter, H. L., *J. Applied Physiol.*, 1951, v3, 681.

4. Kraybill, H. F., Bitter, H. L., and Hankins, O. G., *ibid.*, 1952, v4, 575.

5. Wellington, G. H., Reid, J. T., Bratzler, L. J., and Miller, J. I., *J. Animal Sci.*, 1956, v15, 76.

6. Kraybill, H. F., Goode, E. R., Robertson, R. S. B., and Sloane, H. S., *J. Applied Physiol.*, 1953, v6, 27.

7. Faller, I. L., Petty, D., Last, J. H., Pascale, L. R., and Bond, E. E., *J. Lab. and Clin. Med.*, 1955, v45, 748; *ibid.*, 759.

8. Edelman, I. S., Olney, J. M., James, A. H., Brooks, L., and Moore, F. D., *Science*, 1952, v115, 447.

9. Behnke, A. R., Feen, B. G., and Welham, W. C., *J.A.M.A.*, 1942, v118, 495.

10. Osserman, E., Pitts, G. C., Welham, W. C., and Behnke, A. R., *J. Applied Physiol.*, 1950, v2, 633.

11. Rathbun, E. N., and Pace, N., *J. Biol. Chem.*, 1945, v158, 667.

12. Prentice, T. C., Siri, W., Berlin, N. I., Hyde, G. M., Parson, R. J., Joiner, E. E., and Lawrence, J. H., *J. Clin. Invest.*, 1952, v31, 412.

13. Talso, P. J., Lahr, T. N., Spafford, N., Ferenzi, G., and Jackson, R. O., *J. Lab. and Clin. Med.*, 1955, v46, 619.

14. Hansard, Sam L., US-AEC TID, 1956.

15. Hansard, Sam L., Comar, C. L., and Plumlee, M. P., *Nucleonics*, 1951, v9, 38; *ibid.*, 13.

16. Brodie, B. B., Axelrod, J., Soberman, R. and Levy, B. B., *J. Biol. Chem.*, 1949, v179, 25.

17. Reid, J. T., Wellington, G. H., and Dunn, H. O., *J. Dairy Sci.*, 1955, v38, 1344.

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Characterization of an Antibacterial Peptide from Calf Thymus.* (22728)

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Many investigators have succeeded in extracting antimicrobial substances from various normal tissues during the past several decades. Some of these factors have been shown to be basic proteins or peptides. Bloom *et al.*(1) obtained an anthracidal substance from calf thymus and other tissues. This material was found to be stable to acid and heat, of polypeptide nature, and basic due to the presence of a large amount of lysine. That this antibacterial peptide was derived from a histone has been suggested(16). A mycobactericidal thymus peptide has been described in a series of papers by Dubos and Hirsch (5,7,8). Upon chemical analysis it was shown to be basic, possessing large amounts of lysine and arginine, and having an isoelectric point of 10-11.

This investigation is concerned with further studies and characterization of the basic thymus peptide of Bloom and co-workers(1).

Methods and materials. The tissue peptide was prepared from fresh calf thymus according to the method of Bloom and Prigmore(3) with one modification. The final S-IV product was brought to half saturation with ammonium sulfate at room temperature and pH of 6.0. The supernatant solution was dialysed free of salt and then lyophilized and weighed. Stock solutions of the thymus peptide were made up in distilled water at a concentration of 0.1%. Hyaluronic acid, prepared from type 28 streptococci, was furnished by Dr. Y. Ginzburg of this laboratory, glutamyl polypeptide of the anthrax bacillus was supplied by Dr. R. D. Housewright, and desoxyribonucleic acid was purchased from Nutritional Biochemicals Co. Stock solutions of the various acidic polymers were prepared in distilled water to contain 5 mg/ml. Blockage of antibacterial activity was examined when the acidic macromolecules were added to the test systems just prior to inoculation with the microorganisms. Turbidity readings were made every 2 hours for a 10 hour period, during which time the ability of any particular acidic polymer to reverse growth inhibition could be observed. Normal growth and growth inhibition controls were included with each experiment.

Preliminary experiments were carried out to examine differences in the antibacterial capacity of the thymus peptide against 2 encapsulated and non-encapsulated species. A Group A, type 28 streptococcus and a strain of *Bacillus anthracis* were tested. Thermostability of the peptide was tested in distilled water at acid, neutral and alkaline pH. The pH for greatest activity was determined using phosphate buffered broth at the pH values of 6.7 and 8.0. Electrophoretic mobility of the product was measured using cacodylate buffer (pH 7.0) at an ionic strength of 0.08. An ultraviolet absorption pattern was run using a 0.05% solution of the thymus peptide. An amino acid analysis was performed following the method of Stein and Moore(11,12). The Dowex 50-X4 resin (-400) was used in the column. The thymus peptide sample was weighed from the lyophilized state in the amount of 63.5 mg. The product was hydrolyzed in a vacuum-sealed glass tube with 3.5 ml of constant boiling hydrochloric acid for 48 hours at 110°C. The hydrolysate was carefully washed from the tube into a round bottom flask and vacuum distilled with 3 additions of distilled water. After the third distillation the residue was brought down to approximately a 1 ml volume and carefully washed into a 25 ml volumetric flask with citrate buffer, pH 3.1, and brought to volume with buffer and distilled water. The final pH of the diluted sample was 2.2. The sample was then filtered through a fiber glass pad and stored at 4°C until used. The total nucleic acid content of the thymus peptide was determined from the phosphorus value(6).

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* This investigation was supported by Office of Surgeon General, Department of the Army, and National Heart Institute, N.I.H., Public Health Service.

Desoxyribonucleic acid was measured by a modification of the Dische test(14). The ribonucleic acid value was computed by subtraction of the Dische test result from the total nucleic acids. Total carbohydrate was measured by the anthrone test(13) after deproteinization with trichloroacetic acid, and was based on glucose equivalents. The total protein content of the thymus peptide was obtained from the amino acid assay and by the microKjeldahl method(9).

Results. The *in vitro* antibacterial potency of the thymus peptide was quite high. The final step in the preparation of the active fraction, which involved removal of precipitable materials at half saturation with ammonium sulfate, concentrated activity 2 to 3 times. The outgrowth of a staphylococcus was inhibited for 8 hours by 5 μg thymus peptide per ml of broth.

The acidic macromolecules blocked the antibacterial action of the tissue peptide when the ratio of acid to peptide was equal or greater. The addition of 0.5 to 1.0% NaCl to any of the test systems interfered with the capacity of the acidic polymers to block antibacterial activity of the tissue peptide. In the correlative study with encapsulated and non-encapsulated streptococci and anthrax bacilli it was found that the encapsulated forms were less strongly inhibited by the thymus peptide than the rough forms, particularly in the case of the anthrax organisms. However, the differences in degree of inhibition between smooth and rough streptococci were not always significant.

The peptide was found to be very active at pH 8.0 but its concentration had to be increased 10-fold to approach the same degree of antibacterial effectiveness at pH 6.7. From the electrophoretic determinations the material was found to migrate toward the negative pole at pH 7.0. The mobility was calculated to be 6.8 x 10^{-5} cm^2 volt^{-1} sec.$^{-1}$.

The peptide was active against staphylococci, Group A streptococci and anthrax bacilli but not type II pneumococci. While it inhibited *Escherichia coli, Salmonella typhosa* and *Vibrio comma* were not affected markedly by its antibacterial action. The ultraviolet absorption pattern showed one peak at 264 mμ indicating a protein-nucleic acid complex. The material could be dialyzed in cellophane bags for prolonged periods without a detectable loss in antibacterial potency. It was very heat stable, withstanding boiling temperature for periods up to one hour at neutral or acid pH. However, most of its activity was lost in 15 minutes when boiled at pH 9.6. It was quite potent against a strain of *Micrococcus pyogenes,* var. *aureus* (5-10 μg per ml) in nutrient broth but in more complex media its activity was decreased somewhat. The factor was quite soluble in water at acid or neutral pH, forming tan colored solutions at concentrations of 1-2%.

TABLE I. Amino Acid Analysis of Thymus Peptide.

Amino acid	% amino acid of total wt	g amino acid/100 g protein
Arginine	1.64	2.1
Lysine	27.10	34.7
Phenylalanine	.63	.8
Tyrosine	.34	.4
Leucine	3.20	4.1
Isoleucine	.75	.9
Valine	3.50	4.5
Alanine	13.85	17.8
Glycine	3.48	4.5
Glutamic acid	3.98	5.1
Proline	8.22	10.5
Aspartic acid	2.27	2.9
Threonine	4.10	5.4
Serine	4.24	5.4
Ammonia	.84	1.1
Total	77.25%	99.2

Table I contains the result of the amino acid analysis of the thymus peptide. The basic amino acid, lysine, represented nearly 35% of the total weight of the protein portion. The concentration of arginine was small and histidine was absent. The total protein content of the tissue peptide was found to be approximately 78% by the amino acid analysis. An average value of 80% protein was obtained by microKjeldahl determinations. Hydrolysis of nucleic acids by this latter method may have accounted for the higher value.

From the phosphorus value of 0.69%, total nucleic acids were calculated to be 6.37%. The Dische test result for desoxyribonucleic acid was 4.0% and the ribonucleic acid content, determined by subtraction, was 2.37% of the total peptide. Total carbohydrate, as glucose equivalents, was shown to be 0.83% by the anthrone method.

Discussion. Certain of the characteristics of the thymus peptide which have been studied reaffirmed and enlarged upon the original work which showed that this tissue factor was a basic polypeptide(1). The acid and heat stability of the thymus material has been confirmed. The electrophoretic mobility at pH 7.0 was found to be almost the same as that reported in the original work. The lysine content of the peptide as first reported was 29.3% by microbiological assay and in this case it was 34.7% by column chromatography. Furthermore, thymus peptide antibacterial activity was blocked by the acidic polymers, hyaluronic acid, glutamyl polypeptide and nucleic acid. Such blockage by acidic macromolecules has been reported for the previously cited thymus peptide(2,10, 15). Furthermore, the thymus peptide reported here was found to be most active at an alkaline reaction and apparently more active against gram positive bacteria.

Crampton, Stein and Moore have recently performed amino acid analyses on three calf thymus histone fractions(4). One of the 3, histone Fraction A, was shown to contain a large amount of lysine as well as alanine and proline and to be lacking four amino acids, cystine, methionine, histidine and tryptophane. The thymus peptide studied here was also rich in lysine, alanine and proline and lacking in cystine, methionine, histidine and tryptophane. It seems very likely that this peptide is the same as the histone Fraction A reported by Crampton and co-workers.

The antibacterial thymus peptide investigated by Hirsch and Dubos(7) was shown to contain large amounts of lysine and arginine. From its amino acid analysis it appears that this thymus factor is similar to Fractions B and C of Crampton, *et al.* Since the peptide was dialyzable it is possible that it represented a lower molecular weight histone fraction. It is not difficult to envisage the formation of split histone products differing in molecular weight and amino acid composition when extracting these basic proteins by related chemical procedures. The work of Weissman and Graf(16) indicates that these histone derivatives occur as a result of the splitting of the tissue nucleoproteins.

Summary. The antibacterial thymus peptide has been characterized and strong confirmatory evidence has been presented to support the view that it has been derived from a histone present in thymus tissue.

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1. Bloom, W. L., Watson, D. W., Cromartie, W. J., and Freed, M., *J. Infect. Dis.*, 1947, v80, 41.

2. Bloom, W. L., Winters, M. G., and Watson, D. W., *J. Bact.*, 1951, v62, 7.

3. Bloom, W. L., and Prigmore, J. R., *ibid.*, 1952, v64, 855.

4. Crampton, C. F., Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1955, v215, 787.

5. Dubos, R. J., and Hirsch, J. G., *J. Exp. Med.*, 1954, v99, 55.

6. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

7. Hirsch, J. G., and Dubos, R. J., *J. Exp. Med*, 1954, v99, 65.

8. Hirsch, J. G., *ibid.*, 1954, v99, 79.

9. Kabat, E. A., and Mayer, M. M., *Experimental Immunochemistry,* Thomas, Springfield, 1948, p282.

10. Lewis, G. T., Bloom, W. L., and Smart, C. W., *Cancer Res.*, 1949, v9, 103.

11. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1954, v211, 893.

12. ———, *ibid.*, 1954, v211, 907.

13. Morris, D. L., *Science,* 1948, v107, 254.

14. Seibert, F. B., *J. Biol. Chem.*, 1940, v133, 593.

15. Watson, D. W., and Bloom, W. L., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 29.

16. Weissman, N., and Graf, L. H., *J. Infect. Dis.*, 1947, v80, 145.

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Time and Dose Relationships in Estriol-Estradiol Interaction.* (22729)

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Biologic effects of estradiol can be modified by concurrent administration of a second estrogenic substance. Uterotrophic effect of estradiol in castrate female rats can be diminished by injecting estriol(1). Similarly, estradiol-induced pituitary enlargement can be prevented by simultaneous treatment with weaker synthetic estrogens(2). The former effect is reflected not only by organ weights but also by diminished activity of particular enzyme systems in uterine tissue(3). For interpretation of these surprising effects it should be remembered that the dose-response curve of estradiol, after having reached a maximum, declines to a lower plateau. Thus it remains to decide whether combination of two estrogens might only produce the plateau effect of more than optimal estradiol dosages, before other mechanisms are drawn into consideration. Preceding an increase in tissue dry weight, uterine tissue accumulation of fluid results from estrogenic stimulation. The onset of this fluid uptake phase is more rapid with estriol administration than with estradiol(4). In concurrent injections of both hormones, rapidly absorbed and faster acting estriol may diminish the observed response to estradiol by causing a precocious development of maximal uterine response which would pass unnoticed in a standard 3 day assay.

It is the purpose of this study to investigate the possibility of masked-summation effects of estradiol and estriol. Evidently temporal and qualitative differences in uterine response produced by various hormones may also affect the outcome of combination treatments. An analysis has been made of weight changes of uteri in immature female mice following administration of estradiol, estriol and combinations of both.

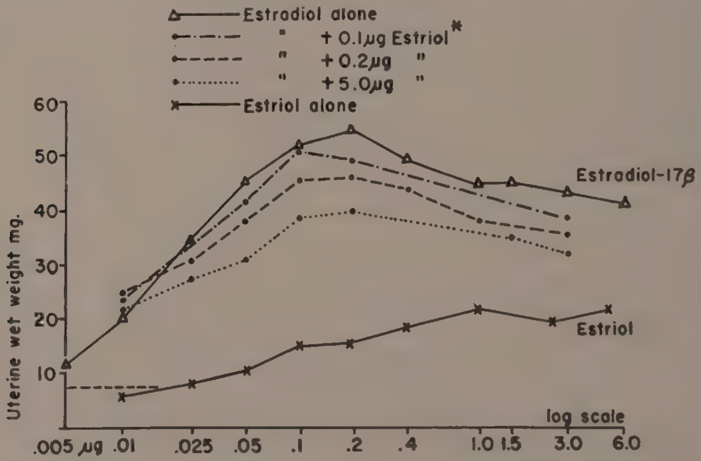

FIG. 1. Dose-response curves of estradiol-17β estriol and combinations of both. The dose of estriol is constant in each of the combination curves, as indicated in the key. Statistical computations appear in Table I.

Methods. Estrogen assays were performed according to the method of Evans, Varney and Koch(5). Dose groups of 5 to 20 immature female mice (Hamilton Laboratory strain) were injected with estradiol-17β, estriol or both, b.i.d. for 3 days. Each total

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TABLE I. Effects of Estradiol-Estriol Combinations on Uterine Wet Weight. 3-day assay.

Estradiol (μg)	Estriol (μg)	No. of animals	Mean	ϵ*	t†	P value
.005	—	14	11.5	.45	—	—
.01	—	24	20.6	.77	—	—
	.01-.1	10	23.7	1.10	2.31	>.02
	.2	5	23.9	1.70	1.77	>.05
	5.0	10	21.3	.74	.66	"
.025	—	10	34.6	1.24	—	—
	.2	10	30.3	1.29	2.40	>.02
	5.0	15	27.7	1.54	3.50	<.001
.05	—	61	45.3	1.14	—	—
	.01-.1	30	41.1	1.23	2.50	>.01
	.2	28	37.4	1.40	4.39	<.001
	5.0	53	30.3	.78	10.87	"
.1	—	42	51.4	1.76	—	—
	.01-.1	30	50.7	1.80	.28	>.1
	.2	20	45.5	2.46	1.95	.05
	5.0	29	38.7	1.90	4.90	<.001
.2	—	22	54.7	2.74	—	—
	.01-.1	30	48.4	1.32	2.07	>.02
	.2	20	45.7	2.14	2.59	>.01
	5.0	39	39.6	1.37	4.92	<.001
.4	—	29	48.5	2.10	—	—
	.2	10	43.4	2.94	1.41	>.05
1.0	—	39	44.5	2.23	—	—
	.2	9	37.1	3.05	1.96	>.05
1.5	—	40	44.9	1.79	—	—
	5.0	5	34.3	3.42	2.75	<.01
3.0	—	40	42.9	1.23	—	—
	.01-.1	10	38.0	2.60	1.70	>.05
	5.0	5	31.4	3.09	3.46	<.01
6.0	—	20	40.7	2.02		

* $\epsilon = \frac{\sqrt{\Sigma d^2}}{n(n-1)}$. † $t = \frac{M_1 - M_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$.

dose was contained in 0.3 cc corn oil solution so that a single injection was a volume of 0.05 cc delivered from a 0.25 cc syringe. When 2 estrogens were administered each was given at a separate injection site. A control group injected with corn oil was included with each experiment. Small volumes used eliminated leakage from the needle puncture. On the fourth day, 18 hours after last injection, animals were sacrificed by cervical dislocation and uteri were freed at vagina and caudal ends of the oviducts. Uteri were blotted dry and weighed on a 50 mg Roller-Smith torsion balance. For each group receiving estradiol or mixed estrogens the mean and its standard error were calculated. Student's t test was applied using the formulae indicated in Table I, and employing the 1% confidence limit for determining significance. In some cases means represent the combined data of several experiments. Number of animals per group and pertinent statistical computations appear in Table I. In a separate set of experiments injection schedules were set up as above with 30 animals per group. At the end of 1 day, 2 days, and 3 days, 10 animals of each dosage group were sacrificed and uterine weights recorded (Table III). Dry weights of uteri were obtained by placing organs in preweighed aluminum foil pans and drying in an oven at 100°C. Usually constant final dry weight was achieved after 18 hours.

Results. Dose-response curves of estradiol and estriol (*Fig. 1*). A uterine weight increase of 100% above control value is produced with about .01 μg of estradiol (total 3 day dose). Maximal response occurs only with a total dose of .2 μg. At higher doses uterine weight increase remains below this maximal value. Estriol stimulates 100% increase in uterine weight at a dose of about .1 μg and an even greater increase with a total dose of 1.0 μg. Higher doses of estriol, up to 5.0 μg, do not enhance uterine stimulation. Both dose-response curves, therefore, ascend to maxima and then plateau. Slope of the ascending portion is steeper on the estradiol than on the estriol curve.

Dose-response curves of estradiol and estriol combinations (*Fig. 1*). Estriol inhibits the uterine response to estradiol at all dosage levels tested except .01 μg. At this point there is no significant difference between estradiol alone and any of the combination doses (Table I). Extent of inhibition increases in proportion to the estriol fraction. As a result there is a gradual decline in slopes of mixed dose-response curves; the greater amount of added estriol, the greater decline. In no case does the addition of estriol enhance the estradiol effect.

Effects of estrogen combination on uterine dry weights (*Table II*). The inhibiting effect described above, measured by weights of freshly dissected uteri, is not merely a matter

TABLE II. Effect of Estriol on Uterine Response to Estradiol Dry Tissue Weights—3 Day Assay.

Group*	Dose, μg	Dry wt uterus, mg
Control		1.64 ± .23†
Estriol	5.0	4.23 ± .31
Estradiol	.05	7.33 ± .34
Both	as above	5.46 ± .41‡

* 20 animals/group.
† ± stand. error.
‡ P value, vs. estradiol alone, .001.

of changed water balance but is also reflected by changes in dry weights. This is illustrated by combination of 5.0 μg estriol with .05 μg estradiol, which produces a significantly smaller gain than occurs following administration of .05 μg estradiol alone.

Daily increments in uterine weights with estrogen treatments (*Table III*). Daily uterine wet weight gain during administration of a total dose of .05 μg estradiol over a 3 day period is shown in Table III. Largest percentage gain occurs during the second day of treatment and a further uterine weight increase takes place during the third day. Uterine weight changes induced by a total dose of 5.0 μg estriol in 3 days follow a different course. After a slight initial gain during the first day, major portion of weight increase occurs during the second day and the third day's treatment fails to cause a further increase.

Uterine weight gain of each 24 hour period during the 3 day estradiol treatment is inhibited when estriol is given concurrently (doses of each as above). Uteri of doubly injected animals sacrificed after the first day show only 73% of weight gain resulting from estradiol alone (an inhibition of 27%). For a 2 day period expected estradiol stimulation is diminished by 42%. After completion of the 3 day injection schedule inhibition of weight gain is also 42% (Table III).

TABLE III. Daily Effects of Estrogens on Uterine Weight.

Group*	Dose, μg	Hr	Wet wt uterus, mg	Inhibition of daily wt gain, %†
Estriol	1.66	24	10.1	
Estradiol	.16	"	15.4	
Both	as above	"	13.2	27
Estriol	3.33	48	17.6	
Estradiol	.033	"	34.1	
Both	as above	"	21.9	42
Estriol	5.0	72	18.9	
Estradiol	.05	"	49.2	
Both	as above	"	30.0	42
Control		"	7.3	

* 10 animals/group.
† Estradiol-induced wt gains above control, considered 100%.

Discussion. Impeding effect of estriol occurs along the ascending segment of the estradiol dose-response curve as well as with higher doses. Extent of inhibition, using a conventional 3 day assay, is proportional to amount of estriol administered. There is no instance of an additive effect. From these observations it may be concluded that diminution of uterine weight increase is not a masked-summation effect in which two estrogens exceed the maximum estrogenic response dose. Among the dosages of estradiol employed, only .01 μg gives a response comparable to that obtained with estriol. It is of particular interest, therefore, to compare this dose of estradiol alone with a combination using an amount of estriol which causes the same uterine response (5.0 μg). In this case responses to single estrogens or to the combination are the same. These facts concerning dose relationships of estriol-estradiol interaction suggest that a competitive inhibition results in the attenuated response. Day by day analysis of uterine changes in doubly injected animals supports this view. There is no indication that combination of estriol and estradiol shifts time of maximal effect since inhibition occurs as early as the first day of treatment. Hence doubly injected animals never equal or exceed uterine response resulting from only estradiol.

In addition to estriol, several other steroids have been found to exert a limiting effect on estradiol-induced uterine stimulation in rats (6,7). Particular emphasis has been placed on the ability of adrenal cortical steroids to impede uterine response to estradiol(8). The possibility remains to be tested that estriol and other non-adrenal steroids known to inhibit estradiol stimulation of uterine growth and enzyme systems(3) act by causing release of ACTH and secondarily adrenocorticosteroids with their modulating effect on uterine metabolism.

Summary. Uterine response to estradiol in immature female mice is inhibited significantly by concurrent injection of estriol. Extent of inhibition is proportional to amount of estriol added. Impeding interaction occurs along the ascending segment of the estradiol dose-response curve and with higher doses as well. In no case does a combination of estrogens have a positive additive action. These conclusions are based on both wet and dry uterine weight observations. Day by day analysis shows that uterine weight gain of each 24 hour period during 3 day estradiol treatment is inhibited when estriol is given concurrently. These studies negate the possibilities that estriol-estradiol interaction is a masked-summation effect in which time of maximum effect has shifted, or that an additive effect occurs in which two estrogens combined exceed maximum response dose. Rather, it appears that estriol competes actively at a physiologic site essential in development of usual estradiol stimulation of the uterus.

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1. Hisaw, F. L., Velardo, J. T., and Goolsby, C. M., *J. Clin. Endocrin. and Metab.*, 1954, v14, 1134.

2. Segal, S. J., and Thompson, C. R., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 623.

3. Villee, C. A., *J. Clin. Endocrinol. and Metab.*, 1956, v16.

4. Astwood, E. B., *Endocrinology*, 1938, v23, 25.

5. Evans, J. S., Varney, R. F., and Koch, F. C., *Endocrinology*, 1941, v28, 747.

6. Velardo, J. T., Hisaw, F. L., and Bever, A. T., *Anat. Rec.*, 1953, v117, 552.

7. Szego, C. M., and Roberts, S., *Amer. Jour. Physiol.*, 1948, v152, 131.

8. Velardo, J. T., and Sturgis, S. H., *J. Clin. Endocrinol. and Metab.*, 1956, v16, 496.

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Factors Influencing Host-Virus Interactions. II. Alteration of Coxsackie Virus Infection in Adult Mice by Cold.* (22730)

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Coxsackie virus infections in infant and adult mice are remarkably dissimilar(1,2,3). Many tissues, such as brain, muscle, liver, and heart, develop extensive lesions in very young animals but become refractory to the virus as mice reach 1 to 3 weeks in age. In infections with Conn. 5 strain of Coxsackie virus in adult mice the pancreas appears to be the only regular site of virus multiplication and histopathology. Even though, as a result of initial viremia, virus is available to other tissues, viral multiplication and tissue damage remain localized and non-fatal. A single injection of cortisone alters the infection with Conn. 5 virus in adult mice in such a manner that the infection becomes lethal (4) with multiplication of the virus and histopathology occurring in tissues that are unaffected in untreated animals of the same age (3). This report presents data indicating that the natural resistance of adult mice to disseminated infection with this virus can be altered by another factor, namely a cold environment, to result in rapidly and uniformly fatal disease.

Materials. Virus. The Conn. 5 strain of Coxsackie virus was used and methods for preparation of virus suspensions, normal mouse tissue suspensions, and general methods of virus titration and neutralization tests have been described(3). In this study all infant mice 24-48 hours of age employed in a set of virus titrations were mixed and redistributed to mothers at random, and equal numbers, usually eight, were inoculated with each dilution. Unless otherwise stated, mice referred to as adult mice were 4-6 weeks of age and averaged 14-18 g in weight. Sexes were mixed at random. Immune serum was prepared by injecting mice once weekly for 3 weeks with 0.2 ml of a 1% suspension of infected infant mouse tissue. The mice were bled one week after the last injection. Normal serum was obtained from similar, but uninoculated, mice. A room varying in temperature between 23° and 27°C was used to house mice designated as held at 25°C. There was no special control over humidity which varied with atmospheric conditions. Mice referred to as held at 4°C were placed in a refrigerated room which was maintained at 3-5°C and approximately 60% relative humidity while those designated as held at 36°C were kept in room maintained at 35-37°C and approximately 12% relative humidity. Mice were housed in wire cages open to the atmosphere except for the top, bottom and the lower two inches of the sides, and were caged in groups of 8 to 12 without restrictions on huddling or activity. Food and water were available *ad libitum.*

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* Opinions expressed in this article are solely those of the authors and do not necessarily reflect viewpoint of Navy Department.

† Data in this report were submitted to Graduate School, University of Wisconsin, in partial fulfillment of requirements for degree of Doctor of Philosophy. Present address: Department of Medical Microbiology and Public Health, Medical College of Georgia, Augusta.

Results. Although adult mice of strain used in this study seldom suffer apparent ill effects from even very large doses of the Conn. 5 strain of Coxsackie virus, experiments indicated that if infected mice were kept at 4°C following inoculation, the infection became uniformly lethal. A representative experiment is presented in Table I. Adult mice previously housed at 25°C were inoculated i.p. with 4000 infant LD_{50} of virus and then maintained at test temperatures along with control mice injected with normal mouse tissue suspension. By the end of fourth day all infected mice at 4°C were dead, while infected mice at 25°C, with one exception, survived the 10 day observation period. The one mouse dying in the 25°C groups represents the rare death seen in adult mice infected with the Conn. 5 strain. Although the infection was lethal at 4°C, infected mice placed in another stressing environment, 36°C, did not succumb. Indeed, more animals died in the control than in the test groups at this temperature. This is in keeping with numerous experiments which showed that mice of the strain employed thrived at 4°C for periods up to 3 weeks but that 36°C was a more unfavorable environment and occasional deaths occurred after one week of exposure.

TABLE I. Lethal Effect of Coxsackie Virus (Conn.-5 Strain) Infection in Adult Mice at 4°C.

Inoculum (i.p.)	Environmental temp., °C	Deaths†/No. inoculated
Virus*	25	1/12
	4	10/10
	36	0/ 8
Normal mouse tissue suspension (.05 cc)	25	0/12
	4	0/ 8
	36	3/ 8

* 4000 LD_{50} (.05 cc) for infant mice.

† No. of mice dying during 10 days of observation.

To determine size of viral inoculum required to produce a lethal infection in adult mice at 4°C, comparative titrations of a virus suspension were made using adult mice held at 4°C and 24-48 hr old infant mice at 25°C. The inoculum for both groups was 0.05 ml given i.p. The LD_{50} for adult mice at 4°C was $10^{-4.5}$ and was 10 times the $LD_{50}(10^{-5.5})$ for 24-48 hr old infant mice.

Additional experiments showed that lethal infection followed subcutaneous as well as intraperitoneal inoculation of virus in mice held at 4°C and that alteration of Coxsackie virus infection by a cold environment was not limited to mice 4-6 weeks of age since mice 11 weeks of age were also susceptible. The relative susceptibility of adult mice of various ages was not examined in detail.

Specifically to relate this phenomenon to an activity of the virus in infected mice held at 4°C it was important to determine whether the lethal effect could be neutralized *in vitro* with specific antiviral serum and whether pas-

TABLE II. Specific Neutralization of Coxsackie Virus and Passive Immunization of Adult Mice against Lethal Infection at 4°C.

Preliminary* inj.	Inoculum (i.p.)	Environmental temp., °C	Deaths/ No. inoc.
None	Virus† + antiserum	4	1/10
”	” + normal serum	”	9/10
Antiserum	Virus, 140 LD_{50}‡	”	0/15
”	” 280 ”	”	0/15
Normal serum	” 140 ”	”	13/13
” ”	” 280 ”	25	0/15

* 0.5 cc mouse serum given i.p. 3 hr before inoculum.

† 280 LD_{50} of virus in 0.1 cc of mouse serum.

‡ Determined in infant mice.

sive immunization of mice with specific antiserum would confer immunity to the lethal effect. Data are presented in Table II which show that death at 4°C was prevented by mixing the viral inoculum with specific immune serum prior to inoculation, and by passive immunization of mice with specific antiserum previous to viral infection.

Since it appeared possible that the lethal effect on infected mice at 4°C might have been due only to inability of mice with pancreatitis to survive in the cold, measurements of levels of virus reached in these animals were made, and the pathological lesions produced were compared with those seen in adult mice at 25°C. In Table III are presented the virus levels found in the blood and liver on the 2nd and 4th days after adult mice were inoculated with Conn. 5 virus and then maintained at either 4°C or 25°C. It is evident that in mice at 4°C a viremia persisted through the 4th day although the blood was cleared of virus by that time in mice at 25°C. In the livers of mice in the cold the virus titer increased from the 2nd to the 4th day and was found at a high level on the 4th day of the infection while in mice at 25°C the virus had been reduced to a low level in the liver by the 4th day. Virus recovered from the livers of mice at 4°C was identified as the Conn. 5 strain of Coxsackie virus by neutralization tests in infant mice.

TABLE III. Levels of Virus in Tissues of Adult Mice Infected with Coxsackie Virus (Conn. 5)* and Held at 4°C or at 25°C.

Tissue	Day of infection	Virus titers† in mice at: 4°C	25°C
Blood	2	$10^{-4.5}$	$10^{-4.8}$
	4	$10^{-4.0}$	$<10^{-1.0}$
Liver	2	$10^{-4.0}$	$10^{-3.8}$
	4	$10^{-5.1}$	$<10^{-1.0}$

* Inoculum = 200 infant LD_{50} of Conn. 5 virus.

† Titers in pooled tissues of 5 adult mice as measured in suckling mice.

On gross examination of mice dying of Coxsackie infection at 4°C marked inflammation of perinephric, mesenteric, and pelvic fat was noted in many. In approximately 25% of the animals there appeared to be gross bleeding into the small intestine. Similar abnormalities were not found in infected mice held at 25°C nor in uninfected control mice maintained at 4°C. Microscopic examination of tissues revealed the following: *Lungs.* A slight to moderate interstitial pneumonitis was found in infected animals at both 4° and 25°C. *Heart.* In 7 of 9 infected mice held at 4° lesions were found in the myocardium. These consisted of focal areas of fiber degeneration and some of these foci contained basophilic granules. Occasionally small inflammatory granulomas consisting of fibroblasts, macrophages, and lymphocytes were seen. In contrast to these lesions observed in mice at 4°C, no myocardial lesions were found in infected mice held at 25°C. *Pancreas.* A severe pancreatitis was found in infected mice in both 4°C and 25°C groups. *Fat.* Marked enzymatic necrosis was present in the perinephric and peripancreatic fat in both the 4°C and 25°C groups of animals. Inflammatory lesions in the interscapular fat pad were similar in extent and kind in infected mice at both temperatures. *Liver.* In animals at 25° there were occasional small intralobular and portal inflammatory foci. In mice at 4° similar but more extensive lesions were found and with greater frequency. Additional features in the animals at 4° were considerably cloudy swelling, single or small groups of balloon cells, small areas of parenchymal cell necrosis and, in one animal, marked nuclear pleomorphism associated with slight to moderate numbers of mitoses. *Skeletal Muscle.* No specific abnormalities were found in skeletal mus-

TABLE IV. Relationship of Time of Exposure to 4°C and Lethal Effect of Coxsackie Virus (Conn.-5) in Adult Mice.

Environmental temperature		
Before inoculation*	After inoculation†	Deaths/No. inoculated
4°C for 2 days	25°C	0/12
" " 1 day	"	0/12
25°C	4°C, 1 day, then 25°C	0/12
"	" 2 days, "	0/12
"	4°C	12/12
"	25°C, 1 day, then 4°C	12/12
"	" 2 days, "	9/12
"	" 4 days, "	9/12
"	" 6 days, "	0/12
"	" 8 days, "	0/12

* Inoculum = 1500 infant LD_{50} given i.p. in 0.05 cc.

† Observation period = 10 days after mice were placed at 4°C.

cle at either 25°C or 4°C. *Intestine.* In all mice at 25° and most animals at 4°C no abnormalities were found in the piece of small intestine obtained for histological examination. In 2 of 9 mice at 4°C large quantities of blood were visible in the intestinal contents, confirming the gross examination finding of intestinal hemorrhage. The intestinal mucosa in one of these mice was markedly disrupted suggesting that the site of bleeding was in the small intestine. *Other tissues.* No lesions were found in the brain, kidneys, adrenal, or spleen at either temperature. No abnormalities were found in the tissues of control groups of mice injected with normal mouse tissue extracts and held at 4°C or at 25°C.

Relationship of time of exposure of mice to 4°C and lethal effect obtained with the Conn. 5 Virus. Since an environmental temperature of 4°C was effective in influencing the infection when continued exposure was begun immediately after inoculation, it was desirable to determine the time, relative to inoculation, during which cold would alter the infection and the duration of exposure necessary to result in lethal infection. In Table IV data from one of several experiments designed to explore these points are presented. Exposure of mice to 4°C for periods up to 48 hours either before or after virus inoculation was insufficient to result in fatalities. However, continued exposure of the animals to cold begun as late as 4 days after virus inoculation resulted in the death of the majority of the mice while mice survived if cold exposure was delayed until 6 or more days after infection.

Discussion. The experiments described indicate that although adult mice possess a natural resistance to the Conn. 5 strain of Coxsackie virus such that the disease is limited to a non-fatal infection of the pancreas, this refractoriness is lost when the animals are maintained in a cold environment and a lethal infection results which is characterized by a persisting viremia, high levels of virus in the liver, and lesions demonstrable in other organs as well as in the pancreas. The mechanisms by which cold reduces resistance to this virus are not yet clear although the fact that cortisone causes a similar loss of resistance suggests that cold may act through its capacity as a stress agent to cause excessive secretion of adreno-cortical steroids. That the change in resistance is due only to the non-specific effects of stress might be questioned, however, since heat, also a severe stress, does not reduce resistance. Another possibility presents itself in the well known stimulation of thyroid activity by a cold environment and depression of that activity by elevated temperatures. Evidence that changes in thyroid activity can influence resistance to bacterial and viral infection has been obtained by several investigators(5,6,7) although the direction of the change has not been constant with the several agents studied.

Reports in the literature(8,9,10) indicate that a cold environment may affect other host-virus systems although none so sharply as that studied in the experiments reported here. Cold has usually intensified the results of the infection but in some instances has afforded protection to the host. Because the effects of cold on the Conn. 5 strain Coxsackie virus infection in mice are so sharp and unequivocal we are continuing the study of this infection in an effort to further clarify the mechanisms involved.

Summary. 1. Exposure of adult mice infected with the Conn. 5 strain of Coxsackie virus to a cold environment resulted in an infection with a persisting viremia, high levels of virus in the liver, pathologic lesions in other organs as well as in the pancreas, and a uniformly fatal outcome. 2. A lethal infection was prevented by neutralization of the virus or passive immunization of adult mice with specific antiserum. 3. The LD_{50} for adult mice at 4°C was approximately ten times that for suckling mice at 25°C. 4. It was demonstrated that to initiate a fatal infection exposure to cold had to be continuous and begun within a few days after virus inoculation.

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1. Dalldorf, G., *Bull. New York Acad. Med.*, 1950, v26, 329.

2. Pappenheimer, A. M., Kunz, L. J., and Richardson, S., *J. Exp. Med.*, 1951, v94, 45.

3. Boring, W. D., Angevine, D. M., and Walker, D. L., *ibid.*, 1955, v102, 753.

4. Kilbourne, E. D., and Horsfall, F. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 135.

5. Holtman, D. F., *Science*, 1946, v104, 50.

6. Weiss, E., Moulder, J. W., and Itatoni, M. K., *J. Inf. Dis.*, 1952, v90, 21.

7. Smith, J. M., and Dubos, R. J., *J. Exp. Med.*, 1956, v103, 119.

8. Briody, B. A., Cassel, W. A., Lytte, J., and Fearing, M., *Yale J. Biol. and Med.*, 1953, v25, 391.

9. Griffin, T. P., Hanson, R. P., and Brandly, C. A., *Proc. Am. Vet. Med. Assn.*, 1954, p192.

10. Teodoru, C. V., and Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 181.

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Effects of Short Chain Fatty Acids on Hepatic Acetate Metabolism in Cold Exposure and Fasting.* (22731)

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We reported that liver slices prepared from rats fasted for a period of 24 hours at an environmental temperature of 0-2°C ("cold-fasted" rats) showed severe depression in oxidation of acetate-1-C^{14} to $C^{14}O_2$(1). This defect in acetate oxidation could be restored to normal by addition of glucose(1) or pyruvate(2) to the incubation medium. The evidence suggested that this effect of glucose and pyruvate must be related to a metabolic event prior to entry into the Krebs cycle because succinate was much less effective than pyruvate and glucose in reversing this metabolic defect while citrate and α-ketoglutarate were without effect at all.

Since phases of carbohydrate metabolism other than the Krebs cycle are involved, it is important to know whether acetate oxidation is specifically linked to carbohydrate metabolism or whether other substances can serve as energy sources capable of reversing the block in acetate oxidation imposed by cold exposure and fasting. The latter possibility was tested by adding short chain fatty acids to the incubation medium. The results of these experiments are reported below.

Methods. Adult male rats of Wistar strain were allowed to eat, *ad libitum* for 7 to 10 days, a diet composed of 25 g casein, 10 g fat, 51 g dextrose, 3 g salt mix(3), 5 g cellulose, 6 g Brewer's yeast, 0.04 g cod liver oil concentrate and 0.01 g α-tocopherol. Following dietary stabilization the rats were either given the same diet, *ad libitum*, at 25°C or were fasted for 24 hours at 0-2°C. The animals were killed by blow on head. The liver was rapidly excised and placed in a beaker containing ice-cold Krebs-Henseleit bicarbonate buffer solution(4). Tissue slices were prepared free-hand with razor blade and these slices were collected in petri dish containing ice-cold buffer. The liver slices were blotted free of excess solution on filter paper. Approximately 250 mg of tissue were weighed out and then transferred to specially designed

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† Predoctoral Fellow of National Science Foundation.

incubation flasks(5). All incubations were carried on in duplicate flasks. The main compartment of the incubation flasks contained 250 mg of liver slices, 4.5 ml of Krebs-Henseleit bicarbonate buffer and 0.5 ml of the solution containing the acetate-1-C^{14} as substrate, at concentration in all cases of 1 x 10^{-3} M. When unlabeled substrates were also present, they were neutralized to pH 7.4 with sodium hydroxide and dissolved in the buffer. The system was incubated for 3 hours at 37.5°C with continuous shaking. Total lipids (fatty acids and unsaponifiable materials) were extracted from liver slices by the method of Baruch and Chaikoff(5). An aliquot of the chloroform extract (containing the total lipids) was used for determination of cholesterol digitonide using the method of Sperry and Webb(6). The total C^{14} content of fatty acids was calculated by subtracting the C^{14} content of the cholesterol digitonide from the C^{14} content of the total lipid fraction. The C^{14} content of the lipids and the cholesterol digitonide was determined by methods already described(7). The $C^{14}O_2$ formed during the incubation period was collected by the method of Chernick *et al.*(8) and counted in the form of $BaC^{14}O_3$ by the method of Entenman *et al.*(9). The acetate-C^{14} content was determined by methods described in an earlier paper(7). The unlabeled short chain fatty acids were purchased from commercial sources and were of the highest purity obtainable. Each was further purified by fractional distillation; a Widmer fractionating column was used in these distillations.

Results. The term "control" rat designates rats allowed to eat *ad libitum* at 25°C while "cold-fasted" rat describes rats fasted at 0-2°C for 24 hours.

Liver slices from control rats oxidized 34 ± 0.7% of the acetate-1-C^{14} to CO_2, converted 6.7 ± 0.84% to fatty acids and 0.5 ± 0.07% to cholesterol (Table I). In the case of experiments with control rats, the liver slices were prepared from the same group of rats that were used for the "cold-fasted" experiments and the slices were incubated at the same time and under identical conditions as those of the "cold-fasted" rats to be described below.

TABLE I. Acetate Metabolism by Liver Slices from Control Rats.

No. of rats	% conversion* of acetate-1-C^{14} to		
	CO_2	Fatty acids	Cholesterol
15	34 ± .7†	6.7 ± .84	.5 ± .07

* % of acetate-1-C^{14} converted to CO_2, fatty acids and cholesterol is calculated for a system consisting of 500 mg of liver slices in 10 ml of buffer solution containing 1 × 10^{-3}M sodium acetate-1-C^{14}; the incubation flasks were shaken in water bath at 37.5°C for 3 hr.

† Stand. dev. of mean.

The effects that certain short chain fatty acids have on hepatic acetate metabolism of the "cold-fasted" rats are recorded in Table II. Liver slices from the same rat were incubated with and without the further addition of unlabeled substrate in concentrations noted in this table.

Propionate was added to the incubation medium at 4 x 10^{-3} or 8 x 10^{-3} M concentrations; at neither concentration did it restore the capacity of these slices to oxidize acetate-1-C^{14} to $C^{14}O_2$ but rather further reduced the extent of this conversion. Propionate had little or no stimulating effect on the lipogenic and cholesterogenic activity of these tissues.

Addition of unlabeled butyrate also further depressed acetate oxidation of the "cold-fasted" livers rather than stimulating it, and as with propionate there was no effect on the ability to convert acetate to lipids.

Since caproate is an even-numbered short chain fatty acid, it might be expected to have an action similar to butyrate. Such was found to be the case.

It was shown by Atchley(10) that isobutyrate can be converted to propionate by mammalian mitochondrial preparations; we therefore might expect that isobutyrate would behave in a manner similar to propionate. This proved to be true.

Acetoacetate at 4 x 10^{-3} M and 8 x 10^{-3} M concentrations not only did not stimulate acetate lipogenesis and cholesterogenesis, but, unlike the short-chain fatty acids, had no effect on $C^{14}O_2$ production as well.

Discussion. The fact that butyrate and caproate failed to reverse the block in acetate

TABLE II. Acetate Metabolism by Liver Slices from "Cold-Fasted" Rats.

No. of rats	Unlabeled substrate		% conversion* of acetate-1-C^{14} to		
	Compound	Conc. $\times 10^{-3}$M	CO_2	Fatty acids	Cholesterol
4	None	0	8 ± 1.7†	.1 ± .00	.1 ± .04
	Propionate	4	2 ± .6	.1 ± .00	.0 ± .03
4	None	0	8 ± 1.7	.1 ± .00	.1 ± .04
	Propionate	8	1 ± .4	.1 ± .03	.0 ± .03
2	None	0	14 ± 1.0	.1 ± .02	.0 ± .03
	Butyrate	4	4 ± .5	.1 ± .02	.0 ± .02
9	None	0	9 ± .7	.1 ± .00	.0 ± .00
	Isobutyrate	4	3 ± .7	.1 ± .00	.0 ± .00
4	None	0	12 ± 2.9	.1 ± .00	.1 ± .00
	Caproate	4	5 ± .7	.1 ± .07	.1 ± .07
2	None	0	16 ± 4.5	.1 ± .00	.1 ± .07
	Acetoacetate	4	16 ± 4.2	.1 ± .00	.0 ± .00
2	None	0	16 ± 4.5	.1 ± .00	.1 ± .07
	Acetoacetate	8	13 ± 2.6	.1 ± .00	.0 ± .00

* Refer to footnote, Table I. † Stand. dev. of mean.

oxidation by the "cold-fasted" liver strongly suggests that fatty acid metabolism is not linked with acetate oxidation in the way that glucose and pyruvate metabolism are. Since it was found that butyrate is oxidized by the "cold-fasted" liver at high rates (unpublished), it is apparent that merely the lack of an adequate energy source is not the factor in the "cold-fasted" liver responsible for the low rate of acetate oxidation.

We found it particularly surprising that propionate failed to stimulate acetate oxidation in "cold-fasted" livers since it has long been known that propionate is a glucogenic compound capable of forming considerable amounts of glucose and pyruvate. However, it has recently been reported that propionate does not directly form a carbohydrate intermediate but rather that it enters the carbohydrate schema via the Krebs cycle (by forming succinate)(11). We have already shown that Krebs cycle intermediates(2) are not efficient in reversing the block in acetate oxidation of hepatic tissue from "cold-fasted" rats. We have also demonstrated(2) that relatively large amounts of pyruvate and glucose must be metabolized to reverse this block in acetate oxidation; it is therefore probable that neither Krebs cycle intermediates nor propionate are capable of forming sufficient quantities of carbohydrates and carbohydrate intermediates to effect acetate metabolism appreciably.

In each case there was a reduction in $C^{14}O_2$ formation in the presence of short chain fatty acids; a fact which suggested that those compounds may have an inhibitory action on acetate metabolism. This point has been the subject of an extensive investigation and will be considered elsewhere.

Summary and conclusions. Butyrate, isobutyrate, propionate, caproate, and acetoacetate failed to reverse the block in acetate-1-C^{14} oxidation found in liver slices from rats fasted at 0-2°C for 24 hours. It appears that the ability of glucose and pyruvate to increase the acetate oxidation activity of these liver slices is related to a specific step in carbohydrate metabolism rather than solely to the fact that carbohydrates are excellent sources of energy.

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1. Masoro, E. J., Cohen, A. I., Panagos, S. S., *Am. J. Physiol.*, 1955, v180, 341.

2. Masoro, E. J., Panagos, S. S., Cohen, A. I., and Rapport, D., *ibid.*, 1956, v186, 24.

3. Hubbel, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, v14, 273.

4. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, 1932, v210, 33.

5. Baruch, H., and Chaikoff, I. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 97.

6. Sperry, W. M., and Webb, M. J., *J. Biol. Chem.*,

1950, v187, 97.
7. Masoro, E. J., Cohen, A. I., and Panagos, S. S., *Am. J. Physiol.*, 1954, v179, 451.
8. Chernick, S. S., Masoro, E. J., and Chaikoff, I. L., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 348.
9. Entenman, C., Lerner, S. R., Chaikoff, I. L., and Dauben, W., *ibid.*, 1949, v70, 364.
10. Atchley, W. A., *J. Biol. Chem.*, 1948, v176, 123.
11. Katz, J., and Chaikoff, I. L., *J. Am. Chem. Soc*, 1955, v77, 2659.

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Blood Levels of 17-Hydroxycorticosteroids in Hyperthermic Dogs.* (22732)

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That stress produces an elevation in the level of 17-hydroxycorticosteroids has been indicated by a number of publications(1-5). These observations have ranged from noting the decreased quantity of cholesterol present in the adrenal glands of animals subjected to hemorrhage, to direct measurements of plasma 17-hydroxycorticosteroids in animals and man subjected to surgical, bacterial pyrogen, E.C.T., and whole body radiation procedures. Recently Eik-Nes has reported by abstract, that dogs given an intravenous pyrogenic dose of bacterial mucopolysaccharides exhibited prolonged elevations in plasma 17-hydroxycorticosteroids. The adrenal cortical hormone(s) are known to exert considerable influence not only on the bodily economy of fluids and electrolytes, but also on the partition of these substances. Overman *et al.*(6) have shown that in man and the monkey, marked alterations in Na and K distribution result from prolonged exposure to a febrile condition having its origin in the malarious state. It was suggested that this is indicative of an increased level of certain adrenal cortical hormones in the body.

Since little of a correlative nature is known concerning the stressful hyperpyrexic state, efforts have been made in this laboratory to understand the aberrant physiology of fever. The report of one facet of that investigation —concerning alterations in plasma concentrations of 17-hydroxycorticosteroids, of Na and of K—is the principal concern of this communication.

Materials and methods. Twenty-four healthy mongrel dogs of both sexes and weighing between 10 and 22 kg were subjected to exogenous hyperthermia. Before the animals were placed in the hypertherm cabinet but *after* the administration of intraperitoneal pentobarbital sodium (1 ml of 7% solution/5 lbs. body weight) and after placing of the arterial cannulae, control determinations were made of plasma and R.B.C. electrolytes, rectal temperature and plasma steroid concentrations. Na and K were determined by flame photometry. Rectal temperatures were taken 4 inches proximal to the anus using a thermistor probe. Plasma 17-hydroxycorticosteroids were determined by the method of Nelson and Samuels(7). The dogs were exposed to an ambient temperature of 50°C, generated by shielded infrared heat lamps, for periods sufficient to provoke a rise in rectal temperature to 42.5°C. This temperature was maintained for one hour. All blood samples were obtained from a cannulated carotid artery and were heparinized. Ten dogs, treated in a manner similar to that of the fever experiments with regard to anesthesia, minor surgery and elapsed experimental time, were utilized as controls. The survival of experimental animals was found to be less than 10% 24 hours after heating.

Results. The values obtained from the analysis of plasma for 17-hydroxycorticosteroids are presented in Table I. It will be noted

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TABLE I. Control and Experimental Plasma 17-Hydroxycorticosteroid Concentrations (μg %) in Dogs Subjected to Hyperpyrexia.

Control, 37.5°C	39.4°C	At 42.5°C	42.5°C + 0.5 hr	42.5°C + 1.0 hr	1.5 hr after cabinet removal
3		2		5	
2		5		5	
3		6		28	
3	11	8		18	
3	4	3	11	9	
4	9	13	9	11	
7	12	12	14	14	
5	0	7	10	4	
3*	5	8	22	21	
0*	3	5	11	8	
2*	5	5	11	11	
2*	3	3	9	10	
0*	1	3	8	18	
0*		1			
0*		6			
6*				16	
6*				17	
8*				25	
7*				28	36
6*				17	15
4*				16	28
4*				9	12
8*				27	32
2*				17	28
Avg					
4	5	6	12	15	25
% change					
	+25	+50	+200	+275	+525

* Blood removed from these dogs was replaced from donor dogs.

that after the rectal temperatures had been increased from the average control of 37.5°C to 42.5°C and maintained at that level for one hour, plasma cortical steroid level had risen from 4 to 15 $\mu g/100$ ml plasma. Observations were made at the following times during the experimental period: at a point half way between the control rectal temperature and 42.5°C (39.4°C in Table I), after the temperature had reached 42.5°C, after one half hour at 42.5°C, and after one hour at 42.5°C. In addition, plasma steroid concentrations were measured in 6 dogs about 1.5 hours after removal from the cabinet (average rectal temperature, 39.4°C).

Fig. 1 shows that during the first 120 minutes, both experimental and control animals presented increments in circulating plasma 17-hydroxycorticosteroids of 2 and 3 μg % respectively. During this interval the control animals' rectal temperatures decreased an average of 1.5°C from their control value (38.8°C). It is conceivable that the hypothermic tendency produced this increment in the control animals; no comparable decrease in rectal temperature occurred in the experimental dogs. After 120 minutes, however, the course of the 2 groups of dogs became quite divergent, with those exposed to elevated rectal temperatures exhibiting marked increments in circulating plasma 17-hydroxycorticosteroid levels (Fig. 1). It was feared that the bleeding necessary to obtain adequate plasma for electrolyte and 17-hydroxycorticosteroid determinations (approximately 35 ml of whole blood), in combination with marked diminution in circulating plasma volume as reported by Agersborg *et al.*[†] might augment the stress produced by the hyperthermic state. Consequently, the volume of blood removed for analysis in the last 16 animals presented in Table I was replaced from healthy, normal, donor dogs. It will be seen that similar increments in plasma 17-hydroxycorticosteroids occurred despite the replacement.

The average steroid level of the 6 dogs after removal from the hypertherm cabinet was 25 $\mu g/100$ ml plasma in contrast to only 4 μg % in the controls (Table I, Fig. 1). Removal of the external stimulus apparently does not reverse the tendency for increasingly augmented 17-hydroxycorticosteroid levels.

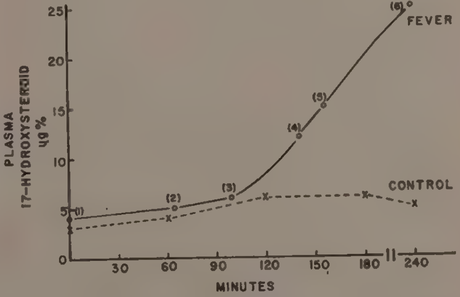

FIG. 1. Each point represents the avg of 6 or more determinations: (1) Avg control rectal temp., 37.5°C. (2) Avg rectal temp., 39.4°C; $p = 0.5$. (3) Rectal temp., 42.5°C; $p = 0.05$. (4) Rectal temp., 42.5°C; $p = < 0.001$. (5) Rectal temp., 42.5°C; $p = < 0.001$. (6) Avg rectal temp., 39.5° C; $p = < 0.001$.

[†] Abstract

Plasma K concentrations were increased from control values of 3.9 to 5.3 mEq/l at the end of the experimental period ($p<0.001$). In general, plasma Na levels were not altered. However, the erythrocyte Na concentration was increased from a control value of 111 mEq/l to an experimental average of 115 mEq/l which is significant at the 1% level. In addition, red blood cell K concentration increased.

Discussion. The data show that severe hyperthermia produces a marked rise in the circulating level of 17-hydroxycorticosteroids in anesthetized dogs. It cannot be ascertained from these data what mechanisms are responsible for such pronounced increments; however, judging from the control determinations, anesthesia, minor surgical trauma and duration of experiment cannot be indicted. Several other possibilities might be suggested.

1) It is possible that thermal stimulation of adrenal cortical function occurs either directly or via ACTH.

2) The rate of hormone destruction in the liver might be impaired. It has been shown by Tyler *et al.*(8) and Sandberg and his colleagues(1) that during surgical stress the adrenal cortex is only submaximally stimulated, and the resulting elevation of corticosteroids results from a decreased ability of the liver to remove 17-hydroxycorticosteroids from the plasma. In this regard two modes of action are distinct possibilities in animals subjected to hyperpyrexia: a) the elevated body temperature might reduce the capacity of liver cells to conjugate 17-hydroxycorticosteroids, and b) the altered circulation of these dogs might contribute to the inability of the liver to handle adrenal steroids in a normal manner. Numerous observations of hemodynamics in hyperpyrexic dogs in this laboratory have shown marked skin dilation associated with measured rises in peripheral resistance. This suggests visceral vasoconstriction which might well decrease liver blood flow. Thus, while liver cells may still be functional or even hyperactive in the face of thermal stimulation, a serious reduction in liver blood flow could result in diminished removal of 17-hydroxycorticosteroids from plasma.

3) There is a possibility that a decreased rate of utilization or destruction could occur in the peripheral tissues of the body, but no means has yet been devised to determine either rate of utilization or destruction of adrenal cortical hormone(s) by tissue cells.

4) The increased plasma steroid levels might result from a concentration effect due to diminished plasma volume. Consideration of a typical animal in which the plasma 17-hydroxycorticosteroid level rose from 6 to 17 μg % during the experimental period reveals that such is not the case. With a control plasma volume of 1015 cc, this dog had 61 μg of circulating 17-hydroxycorticosteroids. At the end of the febrile period, while the plasma volume had been reduced to 791 cc, the total amount of circulating 17-hydroxycorticosteroids equalled 134 μg. If concentration (due to plasma volume reduction) were alone operative, one would expect very little, if any, increment in total circulating plasma 17-hydroxycorticosteroids. The observed increase of 120% in total circulating steroid level cannot be reconciled on the basis of a concentrating mechanism. As a result of these findings, it is suggested that the observed hyper-17-hydroxycorticosteroidemia is due to (1) the possibility of thermal stimulation of the adrenal cortex, (2) diminished rate of hormone destruction in the liver, (3) decreased peripheral tissue utilization, or (4) some subtle combination of these mechanisms.

The electrolyte alterations encountered were all statistically significant at the 2.5% level or lower with the exception of plasma Na concentrations. The marked increase in plasma K concentration ($p<0.001$) is deserving of commentary. Either a concentration mechanism is operative in this instance which is not readily apparent from plasma Na values, or perhaps the plasma K concentration is elevated through the release of adrenaline. Numerous investigators(9-11) have reported that the injection of adrenaline results in the elevation of plasma K concentration, and although the site of release of this K remains unknown, it has been generally attributed to the glycogenolytic influence of

TABLE II. Plasma and Erythrocyte Na and K Concentrations Obtained before and after Dogs Were Subjected to Rectal Temperature of 42.5°C for One Hour.

	Plasma electrolytes, mEq/l				Erythrocyte electrolytes, mEq/l			
	Control		Exp.		Control		Exp.	
	Na	K	Na	K	Na	K	Na	K
	154	3.9	151	6.3				
	154	3.9	151	6.7				
	146	3.9	147	4.0	99	4.2	115	5.0
	141	3.7	144	5.3	106	6.1	118	6.8
	144	4.0	145	5.4	111	6.0	113	6.4
	141	4.2	144	6.2	118	5.4	119	5.7
	146	3.9	144	7.6	115	5.3	116	5.6
	139	3.4	139	4.7	107	6.6	114	5.8
	134	4.0	138	6.0				
	140	3.5	140	4.7	113	4.0	118	4.5
	143	3.7	143	6.8	108	3.9	111	5.0
	148	3.3	145	4.6	115	5.4	113	5.1
	140	4.0	142	5.0	106	5.0	109	5.4
	149	3.8	148	4.7	110	6.1	120	6.2
	150	4.2	151	4.4	118	5.9	107	5.4
	149	4.4	150	4.8	109	6.3	119	6.9
	149	4.2	149	5.1	115	5.7	119	5.5
	150	4.0	149	5.8	112	5.8	115	6.7
	154	3.5	152	3.6				
	143	3.9	145	4.7	112	4.8	118	6.1
	145	4.1	147	4.9	112	5.3	116	5.4
Avg	146	3.9	146	5.3	111	5.4	115	5.8

adrenaline in the liver.

A consideration of 6 dogs in which plasma volume measurements were made shows that no change in total circulating plasma K occurs after the hyperthermic experience. This would appear to offer concrete evidence of a concentrating mechanism. However, in these experiments, transfer of K occurred into erythrocytes (Table II) which may have contributed to the absence of elevated total circulating K. The magnitude of the K uptake by the red blood cells is not too clear from these studies and a more complete treatment must await thorough investigations concerning alterations in the amount of cell ion. Preliminary determinations indicate that the magnitude of increase in the *amount* of erythrocyte K is quite sizeable (c. 190%).

With regard to plasma volume reductions it should be pointed out that the plasma represents the most labile fraction of the body fluid. Since the electrolyte concentrations are essentially similar on either side of the endothelial barrier, the increment in plasma K concentration would be reflected in the interstitial fluid. It seems unlikely, however, that the reduction of the plasma volume would be accompanied by an equivalent decrement in the larger and more stable interstitial fluid compartment. Therefore, the distinct possibility remains that an actual increment existed in the amount of extracellular K in these animals. The point to be emphasized here is that in many instances it is difficult to evaluate the real meaning of electrolyte alterations on the basis of altered plasma concentration alone. Such considerations apply equally to all plasma constituents. Thus, these experiments give us no real measure of the absolute amount of extracellular K, and one can only surmise that it has increased.

With these considerations in mind it would be well to regard the respiratory musculature as a possible source of some of the K. It has been repeatedly shown that muscular activity promotes loss of much cell K, and that such loss is proportional to the strength and duration of contraction(10). All animals used in these studies had very rapid breathing rates (>250/min) many of which exceeded the ability of the investigators to count by palpation, and all of which involved not only the thoracic musculature, but also the accessory respiratory muscles of the abdomen. It is therefore suggested that marked muscular effort might materially add to the extracellular K levels.

Likewise, in the face of dehydration it was noted that no change occurred in plasma Na concentration (Table II). However, the total amount of circulating Na diminished from an average of 114 meq to 91 meq in the 6 animals in which plasma volumes were measured. Clearly Na is being lost from the vascular system and the data presented in Table II would indicate that some of it is making an appearance in the erythrocytes. It is also conceivable that Na is moving into respiratory muscle cells in exchange for K, but such measurements have not been made in this experiment. The transfer of Na into muscle and red blood cells could account for the lack of rise in plasma Na concentration seen at the end of the experimental period instead of an increased concentration as might be expected to result from the loss of water. That increments in erythrocyte Na occurred is clear, and while it is very difficult to assess the importance of alterations in canine red blood cell electrolytes, because their chemical anatomy is quite different from other body cells, such increases could be mediated by the influence of excessive amounts of 17-hydroxycorticosteroids on membrane permeability.

Summary. Twenty-four dogs were subjected to an ambient temperature of sufficient magnitude to elevate rectal temperatures to 42.5°C for one hour. These animals were studied with regard to plasma 17-hydroxycorticosteroid concentration and plasma and erythrocyte electrolyte levels. Significant changes were observed in all measured variables with the exception of plasma Na concentration, which was not altered from the control value. The physiological implications of these data are discussed.

1. Sandberg, A. A., Eik-Nes, Kristen, Samuels, L. T., and Tyler, F. H., *J. Clin. Invest.*, 1954, v33, 1509.

2. Bliss, E. L., Migeon, C. J., Eik-Nes, Kristen, Sandberg, A. A., and Samuels, L. T., *Metabolism Clinical and Experimental*, 1954, v3, 493.

3. Migeon, C. J., Nelson, D. H., Samuels, L. T., and Branch, C. H. H., *Arch. Neurol. and Psychiat.*, 1954, v72, 352.

4. French, A. B., Migeon, C. J., Samuels, L. T., and Bowers, J. Z., *Am. J. Physiol.*, 1955, v182, 469.

5. Sayers, G., Sayers, N. A., Liang, T., and Long, C. N. H., *Endocrinol.*, 1945, v37, 96.

6. Overman, R. R., Bass, A. C., Davis, A. K., and Golden, A., *Am. J. Clin. Path.*, 1949, v19, No. 10, 907.

7. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 519.

8. Tyler, F. H., Schmidt, C. D., Eik-Nes, K., Brown, H., and Samuels, L. T., *J. Clin. Invest.*, 1954, v33, 1517.

9. D'Silva, J. L., *J. Physiol.*, 1934, v82, 393.

10. Fenn, W. O., *Physiol. Rev.*, 1940, v20, 377.

11. Muirhead, E. E., Goth, A., and Jones, F., *Am. J. Physiol.*, 1954, v179, 1.

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Increased Activity of Renal Glutaminases in Guinea Pig Following Prolonged Administration of Acid or Alkali.* (22733)

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The relationship between ammonia excretion and renal glutaminase activity has attracted much attention in recent years. Davies(1) discovered a marked increase in the ability of kidney slices from chronically acidotic rats to deaminate glutamine and other amino acids. On the other hand, Handler(2) found no increased hydrolysis of glutamine in slices from rats chronically treated with acid or alkali. White(3) observed no increase in renal glutaminase activity in homogenates from acidotic rats, while Rector(4) demonstrated a sharp day to day rise in the glutaminase activity of kidney homogenates of rats treated with ammonium chloride. Contrary to observations in man, dog and rat, we observed that, in the guinea pig, not only does ammonia excretion increase in acute acidosis but also in acute alkalosis(5). It was thought that investigation of the activity of renal glutaminases in the two conditions might aid in elucidating the biochemical mechanisms responsible for the increased urinary ammonia excretion. As a basis for work on the enzymatic mechanism of ammonia production, it was mandatory to characterize the enzymes involved. This had not been done previously and might explain the controversial observations. We have been able to distinguish three enzymes involved in glutamine metabolism in

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the renal tubular cells[||]: *Glutaminase I* is a hydrolyzing enzyme with an optimal pH of 7.4, is activated by phosphate and is inhibited by Sulfobromophthalein or Quinacrine (6). *Glutaminase II* is a hydrolyzing enzyme system with an optimal pH of 8.8, is dependent on the presence of α-keto acids, and is similar to the glutamine transaminase-deamidase of the liver studied extensively by Meister(7). *Glutamine synthesizing enzyme system* forms glutamine from glutamic acid and ammonia, has an optimal pH of 7.4 in homogenates, is dependent on adenosinetriphosphate and magnesium ions, and is inhibited by p-chloromercuribenzoate(7). Since it is not known which of these enzymes are involved in ammonia formation, all were investigated in this study.

Materials and methods. Adult guinea pigs, averaging 450 g (400-485 g), in groups of 3 males and 3 females each were treated for a period of 3 weeks as follows: Each group was intubated daily with either 3 ml of 0.9% sodium chloride, 3 ml 1 M ammonium chloride, or 3 ml 1 M sodium bicarbonate. The latter 2 groups received either 0.5 to 1% ammonium chloride or 1 to 2% sodium bicarbonate respectively in their drinking water. All animals were fed Purina Rabbit Chow Checkers supplemented daily with cabbage. The animals were killed by a blow on the head. The kidneys were removed, decapsulated, chilled, and made into a 5% homogenate according to the procedure of Potter(8). The enzyme assays were carried out under optimal conditions as established by previous experiments. The following incubation mixtures were used: *Glutaminase I*: .1 ml 5% tissue homogenate, .1 ml .1 M 1-glutamine. .05 ml 1 M sodium phosphate (pH 7.4), .2 ml .05 M 2-amino-2 - hydroxymethyl-1,3 - propandiol (tris buffer) (pH 7.4), .9% sodium chloride to make 1 ml. *Glutaminase II*: .3 ml 5% tissue homogenate, .1 ml .1 M 1-glutamine, .1 ml .5 M sodium pyruvate, .2 ml .1 M Veronal-HCl buffer (pH 8.8), .9% sodium chloride to make 1 ml. *Glutamine synthesizing enzyme*: .3 ml 5% tissue homogenate, .2 ml .5 M sodium 1-glutamate, .2 ml 2.94 mM ammonium chloride, .1 ml .8 M magnesium sulfate, .2 ml .05 M adenosinetriphosphate-sodium (ATP), .2 ml .05 M tris buffer (pH 7.4), 0.9% sodium chloride to make 1.5 ml. All incubations were carried out in 25 ml serum bottles at 37.5°C for 30 minutes. At the end of the incubation period, the bottles were stoppered, injected through the stopper with 1 ml 20% sodium carbonate and analyzed for ammonia by the micro-diffusion method of Seligson(9). Glutaminase I activity was derived by taking the difference between ammonia liberated in the presence and in the absence of phosphate and corrected for the phosphate-activated, non-enzymatic decomposition of glutamine. For Glutaminase II activity, the assay results in the absence of pyruvate were subtracted from those obtained in the presence of pyruvate. Glutamine synthesis was calculated from ammonia disappearance during incubation and corrected for preformed ammonia and ammonia liberated from endogenous substrates. All results are expressed as μM ammonia liberated per 100 mg desoxyribosenucleic acid, as suggested by Knox(10) since it reflects the number of cells present. The results are similar when wet or dry weight is used as a base but show somewhat greater variability. Desoxyribosenucleic (DNA) and ribosenucleic (RNA) acids were determined on a sample of the original homogenate. The method employed for DNA was a combination of the technic developed by Schneider(11) and of Dische's color reaction for desoxyribose(12). The method of Mejbaum(13) was used to determine RNA.

Results. The percent increase in body weight of the acidotic animals was significantly less than the control or alkali treated groups. The renal DNA concentration was lower in the alkalotic animals than in the controls (Table I). Since the kidney weights were essentially equal, cytoplasmic mass must have been greater in the kidney cells of the alkalotic animals than in the controls. Renal RNA concentration was similar in all 3 groups.

To justify the statement that an increase in enzyme activity is due to induction, the following criteria must be fulfilled(14,15).

[||] Unpublished data.

TABLE I. Effect of 3 Weeks Administration of Acid or Alkali on Body Weight, Kidney Weight and Concentration of Desoxyribosenucleic and Ribosenucleic Acids in Kidney of Guinea Pig.

Group (No. of animals)	Body wt		Kidney wt		Nucleic acids	
	Increase, %	Final, g	% body wt	g	DNA mg/1000 mg d.w.‡	RNA mg/1000 mg d.w.‡
Control (6)	20 ± 3†	533 ± 17	.79 ± .03	4.1 ± .3	45.0 ± 3.2	4.8 ± .3
Acidotic (6)	*10 ± 3	*476 ± 10	.86 ± .04	4.0 ± .2	43.2 ± 3.3	4.6 ± .2
Alkalotic (5)	23 ± 2	528 ± 26	.83 ± .04	4.3 ± .3	*36.9 ± 1.6	4.2 ± .2

* Differs from controls, $P < .05$. † Mean ± S.E. ‡ Dry wt.

First, there must be an increase in enzyme concentration. To rule out the effects of altered permeability, cofactors, etc., homogenates should be employed for assays. Second, to eliminate an increase in enzyme activity due to an increased number of cells, enzyme activity should be expressed on a per cell basis. Since the DNA content of each cell of a species is relatively constant, enzyme activity is best expressed in terms of DNA. Third, since enzyme induction is a form of protein synthesis, it is dependent on amino acid and energy supplies and can be blocked by amino acid antimetabolites or agents blocking oxidative phosphorylation. In the present study the first two criteria have been fulfilled, while the third will be studied in a succeeding investigation.

Increased renal glutaminase activity occurred in the alkalotic as well as in the acidotic group (Table II). Activities of Glutaminase I, Glutaminase II, and the glutamine synthesizing enzyme were elevated in alkalosis and Glutaminase I in acidosis. The scattering of data with reference to Glutaminase I in alkalosis and Glutaminase II in acidosis was conspicuous and may, in some way, be connected with the metabolic alterations produced by the two conditions. The increase of Glutaminase I in acidosis is in agreement with the observations of Davies (1), who employed slices, and Rector(4) who used homogenates of kidneys from rats treated with acid. The contradictory results of Handler(2) and White(3) are probably due to the small number of animals used (only one or two acidotic animals were investigated) and the equivocal assay methods employed (non-optimal conditions).

TABLE II. Effect of Prolonged Administration of Acid and Alkali on Renal Glutaminases.

Group (No. of animals)	Glutaminase I*	Glutaminase II	Glutamine synthesizing enzyme
Control (6)	47 ± 10†	104 ± 9	98 ± 22
Acidotic (6)	§ 96 ± 12	140 ± 30	159 ± 24
Alkalotic (5)	‡141 ± 41	§172 ± 12	‡189 ± 29

* All enzyme activities expressed as μM NH_3 liberated or disappearing (glutamine synthesizing enzyme) from the incubation medium per 100 mg DNA per 30 min.

† Mean ± S.E.

‡ Statistically different from controls ($P < .05$).

§ *Idem* ($P < .01$).

The observation that glutaminase activity in homogenates from alkalotic animals is higher than the controls differs from the observation of Davies(1) that the activity of this enzyme in slice preparations from alkali treated animals was lower than the controls. He, however, employed slices whereas homogenates were used in the present study. Slices not only hydrolyze, but even in the absence of added ATP, synthesize glutamine. Since our studies show for the first time an increased glutamine synthesizing enzyme activity following prolonged administration of alkali, the decreased formation of ammonia from glutamine which Davies found may have been a consequence of assaying for glutaminase in the presence of increased glutamine synthesizing enzyme. Glutaminase II activity, which was observed in the present study to be increased by treatment with alkali, was not studied by Davies.

Whether glutamine from the blood is the only substrate for ammonia production or whether glutamine synthesized intracellularly is an additional source of ammonia is not known. Since both acidosis and alkalosis stimulate the ammonia production and increase the glutaminase activity of the kidney, it is possible that prolonged treatment with either acid or alkali could diminish both blood and renal glutamine stores and increase the glutamate available to the synthesizing enzyme. This in turn would stimulate the synthesis of glutamine by the kidney and lead to increased activity of the glutamine synthesizing enzyme.

Summary. Increased activity of the enzymes involved in ammonia metabolism in the kidneys of guinea pigs was produced by prolonged treatment with acid or alkali. When the results were expressed on a per cell basis, Glutaminase I was increased in acidosis while in alkalosis Glutaminases I and II and the Glutamine synthesizing enzyme were increased. These increases in activity are suggestive of enzyme induction though all the criteria for this process have not yet been fulfilled.

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1. Davies, B. M. A., and Yudkin, J., *Biochem. J.*, 1952, v52, 407.

2. Handler, P., Bernheim, F., and Bernheim, M. L. C., *Arch. Biochem.*, 1949, v21, 132.

3. White, H. L., and Rolf, D., *Am. J. Physiol.*, 1952, v169, 174.

4. Rector, F. C., Jr., Seldin, D. W., and Copenhaver, J. H., *J. Clin. Invest.*, 1955, v34, 20.

5. Richterich-van Baerle, R., Goldstein, L., and Dearborn, E. H., *Science*, 1956, v124, 74.

6. Archibald, R. M., *J. Biol. Chem.*, 1944, v154, 657.

7. Meister, A., *Physiol. Rev.*, 1956, v36, 103.

8. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, v114, 495.

9. Seligson, D., and Seligson, H., *J. Lab. Clin. Med*, 1951, v38, 324.

10. Knox, W. E., *Physiol. Rev.*, 1956, v36, 164.

11. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.

12. Dische, Z., *Mikrochemie*, 1930, v8, 4.

13. Mejbaum, W., *Z. f. physiol. Chem.*, 1939, v258, 117.

14. Knox, W. E., *Brit. J. Exp. Path.*, 1951, v32, 462.

15. Spiegelman, S., Chap. 6, *The Enzymes*, edited by J. B. Sumner and K. Myrbäck, Academic Press, New York, 1950.

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Lipide Analysis of Cellular Fractions in Liver Necrosis.* (22734)

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Within the past few years considerable information has been obtained concerning the pathogenesis of necrotic liver degeneration. It may be produced by dietary(1), chemical (2), or virus(3) means. The biochemical changes occurring in the liver cell during the production of necrosis are probably different for each agent. The necrotic livers of rats fed a *Torula* yeast diet are characterized by a progressive failure of oxygen consumption, ketogenesis, lipogenesis and oxidation of labeled acetate to CO_2(4). Popper *et al.*(5,6) have observed that necrosis caused by bromobenzene administration produces a decrease in the concentration of liver esterase, succinic dehydrogenase and total lipides. However, no analyses were made on the phospholipide content of the liver. Biochemical detoxification of bromobenzene causes depletion of cysteine and its precursors such as methionine from the liver(5). Koch-Weser *et al.*(7) have suggested that the condition produced in the bromobenzene treated rats is an example of a chemical conditional amino acid deficiency. In view of the above findings, experiments were undertaken to determine the lipide content of the various cellular fractions of the liver following bromobenzene administration to ascertain whether the lecithins and cephalins are involved in this process.

Experimental. Male albino rats of the

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Wistar strain weighing 100-110 g were maintained on a Purina stock diet. Acute liver necrosis was produced within 48 hours by the injection of bromobenzene, utilizing the method of Koch-Weser *et al.*(8). Both control and experimental animals were fasted 24 hours. Following the fasting period 11 control rats received an intraperitoneal injection of 1 ml of corn oil. One ml of corn oil containing 0.2 ml of bromobenzene was administered to 15 experimental animals. Forty-eight hours later the rats were sacrificed by decapitation, livers were quickly removed, weighed on a Sartorius Balance and divided into 2 samples, one for histological examination and the other for lipide analysis.

The sample for lipide analysis from each liver was minced in an iced beaker and diluted 10-fold with cold 0.88 M sucrose. The mince was homogenized in a Potter-Elvehjem type tissue grinder with Teflon pestle for approximately 4 minutes in an ice bath, forced through a No. 20 gage hypodermic needle to remove stroma, and centrifuged in a Servall angle centrifuge (Type RR-1), according to a modification of the procedures of Hogeboom *et al.*(9) and Griffiths and Pace(10) for the separation of the cellular fractions by differential centrifugation. Mitochondria Fraction: An aliquot of the homogenate was centrifuged at 1,000 x g for 15 minutes to remove nuclei. The supernatant was then centrifuged at 10,000 x g for 30 minutes and the mitochondria precipitate was washed twice with cold 0.15 M NaCl and finally recentrifuged at 10,000 x g for 20 minutes. The mitochondria fraction was then treated with 10% trichloroacetic acid (TCA) containing 0.4 M $MgCl_2$(11) to remove the acid-soluble phosphorus and was then recentrifuged at 10,000 x g for 10 minutes. The precipitate was washed with 5% TCA containing 0.4 M $MgCl_2$ and recentrifuged at 10,000 x g for 15 minutes. The mitochondria precipitate was then covered with ethanol for dehydration for 6 hours and then washed quantitatively into Soxhlet extracting thimbles with ethanol. Nuclear Fraction: An aliquot of the homogenate was recentrifuged at 10,000 x g for 30 minutes. The precipitate was washed with cold 0.15 M NaCl and 10% and 5% TCA solutions and recentrifuged. The precipitate represents nuclei and mitochondria. The value of the nuclei component was obtained by difference. Homogenate Fraction: The homogenate fraction was precipitated by the addition of 10% TCA, centrifuged at 1000 x g for 15 minutes, washed with 5% TCA and recentrifuged. The lipides from the cellular fractions were extracted with 95% ethanol for 6 hours in Soxhlet continuous extractors. The lipides were purified with chloroform (12). Lipide P(13), cholesterol(14), lecithin and cephalin(15) were determined on aliquots of the chloroform solution. Histological examination showed that the mitochondria and nuclear fractions obtained corresponded in appearance and staining properties with those described by Claude(16) and Hogeboom(9).

Results. The lipide content of cellular fractions from the necrotic and control livers is reported in Table I. The *t* test of significance(17) was applied to the differences between the means for the control and experimental values to evaluate the statistical significance of the results. Histological examination of the liver section showed that necrosis was acute, central, fairly extensive and often confluent, extending between adjacent liver lobules in all specimens. The data in Table I show that bromobenzene necrosis produces a decrease in total lipides, lecithin and cephalin phosphorus in the homogenate fraction, the *t* test being 6.58, 3.90, 3.08 respectively. The probability, P, for chance occurrence of these differences was <0.01. There was a decrease in the lecithin phosphorus of the nuclei of the necrotic livers with a *t* value of 2.15 and $P < 0.05$. The mean values of the total lipide, lecithin and cephalin phosphorus in the mitochondria fraction of the necrotic liver are lower. However, the difference is not significant. The data show that the cholesterol content was significantly greater in the necrotic liver nuclei and homogenate fractions. The mitochondria, nuclei and homogenate *t* values were 1.0, 5.30, 3.61 respectively. The probability, P, for chance occurrence of these differences was <0.01

for the nuclear and homogenate fractions. The rise of cholesterol content in the liver may be the result of increased synthesis, decreased catabolism or excretion. It is of interest that in necrotic liver of animals fed *Torula* yeast there has been observed a decreased conversion of labeled acetate to lipides(4). The pathogenesis of liver necrosis produced by *Torula* yeast is probably different from that of bromobenzene. However, the biochemical changes of lipides in liver necrosis produced by bromobenzene may reflect cellular activity and indicate that there is a lipide disturbance in this process.

TABLE I. Lipide Analysis of Cellular Fractions of Liver 48 Hours following Bromobenzene Administration.*

	mg of P or cholesterol per g of wet tissue											
	Mitochondria				Nuclei				Homogenates			
Exp.	Total lipide P	Lecithin P	Cephalin P	Cholesterol	Total lipide P	Lecithin P	Cephalin P	Cholesterol	Total lipide P	Lecithin P	Cephalin P	Cholesterol
Controls	.25 ± .04	.12 ± .02	.12 ± .02	.39 ± .09	.28 ± .05	.13 ± .05	.14 ± .03	.61 ± .11	1.07 ± .06	.53 ± .06	.46 ± .06	2.92 ± .26
Necrosis	.22 ± .03	.11 ± .02	.10 ± .02	.43 ± .09	.23 ± .04	.09 ± .02	.13 ± .03	1.26 ± .31	.85 ± .07	.42 ± .04	.35 ± .08	3.60 ± .48

* Figures preceded by ± sign indicate stand. dev.

Summary. (1) Lipide analyses of cellular fractions in liver necrosis produced by the administration of bromobenzene was studied in the rat. (2) A statistically significant decrease occurred in the total lipide, lecithin and cephalin phosphorus in the homogenate fraction of the necrotic livers as compared to the controls. However, the total cholesterol content was significantly increased. (3) In the nuclei of the necrotic livers a decrease in the concentration of the lecithin phosphorus and an increase of total cholesterol were noted.

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1. György, P., and Goldblatt, H., *J. Exp. Med.*, 1939, v70, 185; Daft, F. S., Sebrell, W. H., Jr., and Lillie, R. D., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 1; Chernick, S. S., Moe, J. G., Rodnan, G. P., and Schwarz, K., *J. Biol. Chem.*, 1955, v217, 829.

2. Drill, V. A., *Pharm. Revi.*, 1952, v4, 1.

3. Paul, J. R., *Bull. N. Y. Acad. Med.*, 1946, v22, 204; Havens, W. P., Jr., *Med.*, 1948, v27, 279.

4. Rosecan, M., Rodman, G. P., Chernick, S. S., and Schwarz, K., *J. Biol. Chem.*, 1955, v217, 967.

5. Popper, H., de la Huerga, J., Koch-Weser, D , *Annals N. Y. Acad. Sc.*, 1954, v57, 936.

6. Popper, H., Koch-Weser, D., and de la Huerga, J., *J. Mt. Sinai Hosp.*, 1952, v19, 256.

7. Koch-Weser, D., de la Huerga, J., Yesiniek, C., and Popper, H., *Metabolism Clinical and Experimental,* 1953, v2, 248.

8. Koch-Weser, D., de la Huerga, J., and Popper, H., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 196.

9. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, 1948, v172, 619; Schneider, W. C., and Hogeboom, *ibid.*, 1950, v183, 123.

10. Griffiths, M., and Pace, N., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 771.

11. Johnson, R. M., and Dutch, P. H., *ibid.*, 1951,

v78, 662.

12. Artom, C., *J. Biol. Chem.*, 1941, v139, 1953.

13. Fiske, C. H., and Subbarow, Y., *ibid.*, 1925, v66, 375.

14. Kingsley, G. R., and Schaffert, R. R., *ibid.*, 1949, v180, 315.

15. Hack, M. H., *ibid.*, 1947, v169, 137.

16. Claude, A., *J. Exp. Med.*, 1946, v84, 51.

17. Chambers, E. G., *Statistical Calculations for Beginners*, New York: Cambridge Univ. Press, 1952, 2nd edition.

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Oxidation of Unsaturated Fatty Acids in Embryonic and Adult Tissues of Golden Hamster.* (22735)

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Kohn and Liversedge(1) gave evidence that rat tissue homogenates as well as slices incubated aerobically produced a compound which condensed with either p-amino benzoic acid (PAB) or thiobarbituric acid (TBA) and gave a pink color. Bernheim and co-workers showed that the color obtained by Kohn and Liversedge upon addition of TBA to incubated tissues was due to a product of the oxidation of certain unsaturated fatty acids (2). Crystalline hemoglobin and ascorbic acid were found to catalyze the reaction involved. Analysis of the TBA compound showed that a 3-carbon fragment, probably a peroxide, had combined with TBA. Such a reaction, namely, the oxidation of a double bond and the breaking of a fragment containing 3-carbon atoms from the end of the chain could occur in linolenic acid. Wilbur's group suggested the use of TBA as a test for oxidation of highly unsaturated fatty acids, principally linolenic(3). Donnan, working with rat tissues, noted that the results obtained in using the TBA test to estimate the amount of oxidized highly unsaturated fatty acid present in tissue preparations paralleled those obtained by the usual procedure for fat estimation(4). Holman, Lundberg and Malkin(5) reemphasized the TBA test as a means of qualitative and indirect measure of the oxidative products of highly unsaturated fatty acid (*i.e.*, linolenic, linoleic and oleic) content of tissues. Increased concentration of highly unsaturated fatty acids has been associated with increased rate of cell division(6). The following investigation was carried out to compare the oxidation of highly unsaturated fatty acids (HUFA) in terms of their oxidative products (HUFA-p) in brain, testes and liver, tissues of the embryo and young animal, with those of the adult. Experimental procedures were designed also to give evidence for *in vivo* oxidation of highly unsaturated fatty acids. Correlation between HUFA content and development of the tissues, as well as evidence of *in vivo* HUFA-p formation are presented.

Materials and methods. All pregnant females and weaned (15 days) animals derived HUFA's from a diet of Purina rabbit chow checkers supplemented with lettuce twice each week. The analysis of this diet, supplied by the Ralston Purina Co., is shown in Table I. About 50% of the 2.5% fat is made up of unsaturated fatty acids. Entire organs (with exception of adult livers, where only the left lobe was used) were removed immediately from decapitated animals, weighed wet, homogenized at 0-4°C and diluted with phosphate buffer, pH 6.0 to final concentration of 50 mg/ml. One 0.5 ml aliquot of this stock homogenate was added to 1.5 ml of buffer or to a mixture of 1 ml of buffer and 1.5 ml of ascorbic acid (concentration 0.2 mg/ml.) These mixtures contained in 50 ml Erlenmeyer flasks were either incubated in a con-

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* These findings are part of dissertation presented as partial fulfillment of requirements for degree of Doctor of Philosophy.

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TABLE I. Purina Rabbit Chow Checkers

Protein, %	15.00
Fat, %	2.50
Carbohydrate, %	55.00
Fiber, %	16.21
Ash, %	8.04
Calcium, %	1.12
Phosphorus, %	.65
Magnesium, %	.19
Iron, parts/million	161.00
Manganese, " "	55.00
Copper, " "	7.40
Cobalt, " "	.13
Iodine, " "	5.20
Potassium, %	1.03
Carotene (vit. A activity), parts/million	23.00
Thiamin, parts/million	3.70
Riboflavin, " "	7.40
Niacin, " "	38.80
Vit. D, U.S.P. units/g	2.21
Pantothenic acid, parts/million	6.62
Choline, " "	711.00

stant temperature bath for 2 hours at 37° or placed in cold centrifuge tubes and kept in ice bath for not longer than 30 minutes. The latter solutions so refrigerated were considered to represent a measure of the amount of oxidative product of the highly unsaturated fatty acid present in tissue *in vivo* at the time of removal. Values obtained from tissues incubated without ascorbic acid represent levels of HUFA oxidation as influenced by *in vivo* levels of vit. C, hemoglobin and perhaps other unknown protoplasmic catalysts. Total HUFA content of tissues is represented by TBA values for tissues incubated with ascorbic acid added *in vitro*. At end of incubation, or immediately, in the case of unincubated tissues, 1 ml of 20% trichloroacetic acid (TCA) was added and the precipitated protein was removed by centrifugation. To extract the chromogen precursor, at this step bound to the protein in the pellet, 2 ml of 5% TCA was added to the pellet. To the supernatant, and to tubes containing protein and 5% TCA, was added 2 ml of 0.75% thiobarbituric acid. These mixtures were placed in boiling water bath for 15 minutes. All tubes were subsequently returned to total volume of 5 ml with distilled water and the color read and recorded at wave length 525 on Coleman Junior Spectrophotometer. The optical density of supernatant was expressed in terms of Q values read directly from Spectrophotometer. These values were interpreted as a qualitative expression of the fraction of HUFA-p unbound, and values representing color extracted from the pellet were interpreted as a measure of the HUFA-p bound to protein. All values were read against a blank of 2 ml of 20% TCA, 2 ml of 0.75% TBA and 1 ml distilled water. All studies were carried out in duplicate. Values shown in Tables and Figures are in terms of color density produced by 25 mg of tissue and represent total color produced (bound plus unbound fractions).

Results are presented in Figs. 1, 2, and 3. Data on tissues from embryos 14-15 days old are plotted above abbreviation "Emb" and those for tissues from animals within 8 hours *post partum* above the letters "JB". Incubated tissues at every age gave considerably higher values than unincubated tissues. It is also evident that the catalytic action of ascorbic acid is manifest only in incubated tissues. Statistical analysis of unincubated liver and testes tissue data shows no significant difference between values obtained for different aged animals. In brain tissue, however, the values are significantly higher

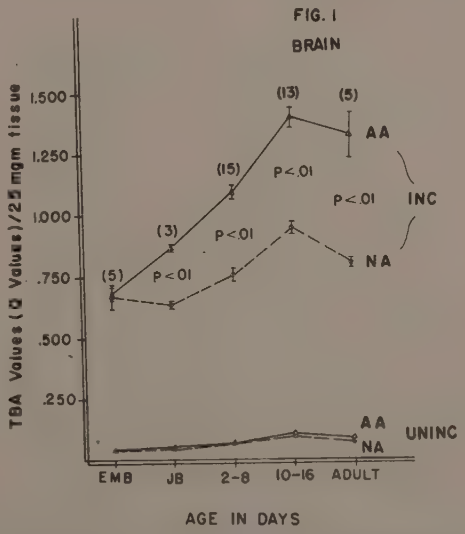

FIG. 1. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in brain tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

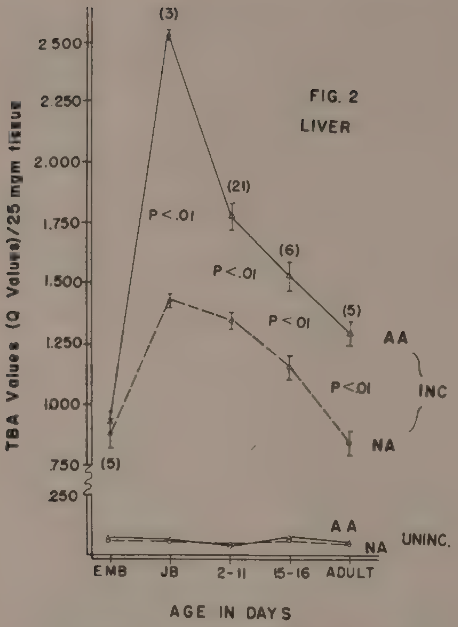

FIG. 2. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in liver tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

($P < .01$) in animals 10 days of age and older, than in embryos or in tissues of animals at birth. Brain values of animals above 10 days old are higher than either liver or testes values, indicating the presence of greater quantities of HUFA oxidized *in vivo* in this tissue.

TBA values for brain tissue incubated with ascorbic acid show significant increases from embryonic tissues through 10 days of age (Fig. 1). Values for tissues of embryo and those of animals through 8 days of age, incubated without ascorbic acid, are significantly lower than values for brain tissue of animals beyond 10 days of age. Catalytic action of ascorbic acid on oxidation of HUFA in embryonic tissue is not apparent.

TBA values for liver tissue incubated with ascorbic acid show a pattern in reverse to that of the brain (Fig. 3 and 4). Although oxidation of HUFA in embryonic liver tissues as in brain, does not reflect the catalytic action of vit. C, values for tissues incubated with ascorbic acid are extremely high at birth and thereafter show a gradual and significant decline to adult levels. The latter are substantially elevated above values for embryonic tissue. Tissues incubated without ascorbic acid indicate a similar trend except that levels in the adult are not significantly different from those in the embryo.

Values for testes tissue incubated with ascorbic acid show an increase from a mean level of .701 in animals 15-30 days of age to a peak of 1.331 in animals 55-65 days of age (Fig. 3). Tissues incubated without ascorbic acid reflect a similar trend with peak in group 35-65 days old.

Discussion. Consideration of TBA values for unincubated liver, brain and testes tissues give evidence of a measurable amount of HUFA oxidized *in vivo* with oxidative products present in tissue at time of sacrifice. These conclusions are based upon the fact that measurable quantities of HUFA-p were found in tissues maintained at low temperature and in consideration of the fact that if oxidation occurred *in vitro* it appears to be a valid assumption that it would be catalyzed by the ascorbic acid added. Bernheim, Wilbur and Kenaston(7) have suggested *in vivo* formation of oxidized fatty acid products may act as normal regulators of some aspects of

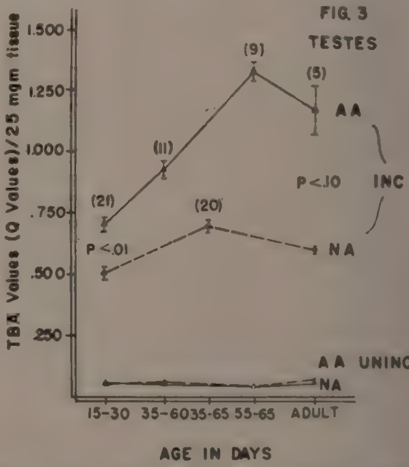

FIG. 3. Mean TBA values which reflect amount of "HUFA-p" available under experimental conditions in testes tissue of different aged hamsters are shown. AA: ascorbic acid added. NA: no ascorbic acid added.

metabolism. Wilbur and co-workers(8) suggest a role of HUFA-p in inhibition of cell division. The oxidative product may well be labile *in vivo* and the values here defined represent those "stabilized" by death of animal and its constituent protoplasm. Except in brain tissue of animals above 10 days old, there is no significant correlation between change of *in vivo* levels of HUFA-p and age. Variation in TBA values of tissues incubated without ascorbic acid reflect *in vitro* oxidation of HUFA in presence of unknown concentration of ascorbic acid which is a constituent part of the tissue under investigation. Therefore, variation in these TBA values may be interpreted as evidence for variation in ascorbic acid content of tissues of animals at various ages, rather than a quantitative expression of HUFA present. Values for tissues incubated with ascorbic acid, under these experimental conditions, probably give a qualitative estimate of total highly unsaturated fatty acid content of the tissue studied.

In the case of brain tissue the content of HUFA gradually rises to that of adult levels in the 10-day-old animal. Since neither the adult nor the embryo is known to synthesize highly unsaturated fatty acids, and in light of the work of Bickenback and Rupp(9) it can be assumed that the only source of HUFA to the embryo is via maternal circulation(10, 11,12). It is significant to note that TBA values in the animals here studied are established at adult levels when the animal is weaned and begins to eat "hard" food, 10-16 days *post partum*. Values for tissues incubated without ascorbic acid do not rise appreciably in the adult, which fact eliminates the probability of an inhibitory action of ascorbic acid added *in vitro*. The abrupt rise in HUFA content of liver tissues correlates well with alteration in liver function known to occur at birth. The findings may be exaggerated by catalytic action of hematopoietic components in the liver during this interval. The gradual rise in total HUFA content of testes tissue from 15 days to a peak in the 55-56 age bracket suggests a significant relationship between fatty acid content, development, and function of the organ. The peak in HUFA content correlates well with age of sexual maturity in the hamster. The gradual, increased difference in TBA values correlated with age, between tissues incubated with and without ascorbic acid suggests a gradual alteration in the *in vivo* content of ascorbic acid.

Perhaps the most significant finding is lack of evidence for catalytic activity of vit. C on *in vitro* oxidation of HUFA constituents of embryonic tissues. This could be due to inhibitory effects of high concentration of ascorbic acid (resulting from high *in vivo* levels), or could be the result of an inhibitory substance peculiar to embryonic tissues and not found in tissues *post partum*. The possible role of variable tissue concentrations of tocopherols and other metabolic antioxidants should be mentioned. Although no bio-assay of vit. E was made, the diet used is sufficient in every respect to sustain our colonies of hamsters indefinitely without showing symptoms of vitamin deficiency. The possibility exists that where TBA values for tissues incubated without ascorbic acid are low, tissues themselves may contain high concentrations of antioxidants not readily identified. Where no difference can be demonstrated between levels of HUFA-p in tissues incubated with and without ascorbic acid (*i.e.*: embryonic tissues) a reaction between some unidentified antioxidant may be manifest. Ability of specific tissues of various aged animals to concentrate tocopherol remains a possibility. Current investigation of this problem is being undertaken.

Summary. The Thiobarbituric Acid Test for oxidative products of essential fatty acids (HUFA), primarily linolenic, present in tissues *in vivo* and after aerobic oxidation *in vitro,* with and without ascorbic acid, was applied to brain, liver and testes tissues of hamsters of various ages. Minute measurable quantities of oxidative products (probably peroxides) are formed *in vivo*. The quantity of HUFA present in brain tissue increased gradually with *post partum* development of the brain, with constant levels apparent after 10-16 days of age. The quantity of HUFA present in testes tissue correlates well with development and function of that organ

with constant levels established, following a peak during age of sexual maturity. There is a significant rise in total HUFA content of liver tissue, associated with emergency of birth. Lack of catalytic activity of ascorbic acid upon *in vitro* oxidative reaction involved in embryonic tissue has been demonstrated.

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1. Kohn, H. I., and Liversedge, M., *J. Pharmacol. and Exp. Therap.*, 1944, v82, 292.

2. Bernheim, F., Bernheim, M. L. C., and Wilbur, K. M., *J. Biol. Chem.*, 1948, v174, 257.

3. Wilbur, K. M., Bernheim, F., and Shapiro, O. W., *Arch. Biochem.*, 1949, v24, 305.

4. Donnan, S. K., *J. Biol. Chem.*, 1950, v182, 415.

5. Holman, R. T., Lundberg, W. O., and Malkin, T., *Progress in the Chemistry of Fats and Other Lipids*, Academic Press, 1954.

6. Decker, A. B., Fillerup, D. L., and Mead, J. F., *J. Nutr.*, 1950, v41, 507.

7. Bernheim, F., Wilbur, K. M., and Kenaston, C. B., *Arch. Biochem. and Biophysics*, 1952, v38, 177.

8. Wilbur, K. M., Kenaston, C. B., Wolfson, N., Ottolenghi, A., and Gaulden, M. E., *Anat. Rec.*, 1954, v120, 25.

9. Bickenback, W., and Rupp, H., *Z. Gebortsh. Gynako.*, 1933, v100, 1.

10. Eckstein, H. C., *J. Biol. Chem.*, 1933, v103, 353.

11. Polonouski, M., Cuvelier, L., and Avenard, R., *Compt. Rend. Soc. Biol.*, 1932, v111, 6.

12. Hughes, E. H., and Hart, H. G., *J. Nutr.*, 1935, v9, 311.

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Influence on Circulating 17-Hydroxycorticosteroid Concentrations of Compounds Structurally Related to Salicylate.* (22736)

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Earlier publications(1,2) reported the finding of marked elevations in circulating 17-hydroxycorticosteroid (17-OHCS) concentrations in guinea pigs following intraperitoneal or oral administration of large doses of sodium salicylate. Hypophysectomy, as well as adrenalectomy, abolished this effect(2). In the present investigation, various other derivatives of benzoic acid were studied with regard to their effects on circulating 17-OHCS concentrations in guinea pigs in an attempt to ascertain whether these effects are related to the structural configuration of the administered compound.

Methods. Male guinea pigs weighing 450 to 750 g were used in these experiments. Sodium salts‡ of the compounds listed in Table I were prepared by dissolving the acids in an equivalent amount of 1 N NaOH; the pH of each solution then was adjusted to neutrality using the Beckman glass electrode. Solutions were diluted with distilled water to contain the equivalent of 100 mg of the acid per ml. Compounds tested were injected intraperitoneally in doses equivalent (on the basis of molecular weight) to 200 mg salicylic acid per kilo body weight. Four hours later the animals were rapidly anesthetized in an ethyl ether atmosphere and bled from the abdominal aorta into heparinized syringes. Additional groups of animals treated with the monohydroxy derivatives of benzoic acid were sacrificed at 1 and 2 hours following injection. Plasmas were separated by centrifugation immediately and stored in deep-freeze until used for analyses. Plasma concentrations of 17-OHCS were determined by the method of Nelson and Samuels(3) as modified by Eik-Nes, Nelson and Samuels(4).

Observations. Data concerning the influence on plasma 17-OHCS concentrations of the various compounds tested are shown in Table I. Benzoic acid had no significant effect. For purposes of statistical analysis the

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† This work was done during tenure of American Heart Assn. Research Fellowship.

‡ For convenience, compounds will be referred to as acids.

TABLE I. Influence of Benzoic Acid Derivatives on Circulating 17-OHCS Concentrations in Guinea Pigs. (Doses equivalent to 200 mg/kg salicylic acid.)

Compound†	No. determinations	17-OHCS* (μg/100 ml)	P	
			vs. benzoic acid	vs. salicylic acid
Untreated	51	37 ± 2.7‡		
Benzoic acid	4	43 ± 5.2		
Monohydroxy derivatives:				
o-OH (salicylic)	5	81 ± 5.6	<.01	
m-OH[1,2]	4	58 ± 9.3	>.05	>.05
p-OH[1,2]	5	57 ± 3.0	>.05	.01–.02
Ether derivatives:				
o-Methoxy[1,2]	7	76 ± 6.7	<.01	
o-Ethoxy[3]	4	119 ± 11.2	<.01	.02
m-Methoxy[2,3]	5	75 ± 11.3	.02–.05	
p-Methoxy[1,2]	5	77 ± 16.7	>.05	
p-Ethoxy[1,2,4]	3	50 ± 2.4	>.05	<.01
3,4,5-Trimethoxy[2,4]	4	119 ± 11.1	<.01	.02
Amino derivatives:				
o-Amino[5]	5	62 ± 4.7	.02–.05	.02–.05
p-Amino (PABA)	3	71 ± 6.9	.02–.05	
Dihydroxy derivatives:				
2,3-OH (o-pyrocatechuic)[4]	3	127 ± 16.6	<.01	.02–.05
2,4-OH (β-resorcylic)[1–4]	9	89 ± 18.6	.02–.05	
2,5-OH (gentisic)[1,4,6]	7	86 ± 11.5	<.01	
2,6-OH (γ-resorcylic)[1,2]	5	123 ± 10.2	<.01	<.01
3,4-OH (protocatechuic)[2]	2	(26, 67)		
3,5-OH (α-resorcylic)[1,4]	4	81 ± 11.6	.02–.05	
Mixed derivatives:				
2-OH, 3-Methyl[1,2,4]	2	(132, 77)		
2-OH, 4-Methyl[2,4]	4	117 ± 14.2	<.01	.05
2-OH, 3-Methoxy[3,6]	7	110 ± 6.8	<.01	<.01
2-OH, 5-Methoxy[2,4]	7	117 ± 18.9	<.01	>.05
2-OH, 4-Amino (PAS)[2]	5	64 ± 16.1	>.05	>.05
Acetylsalicylic acid	7	132 ± 15.2	<.01	.01
Salicyluric acid[2]	4	36 ± 5.9		<.01

* Four hr after intraper. inj. † Each administered as the sodium salt. ‡ Mean ± S.E.

[1] Kindly supplied by Eli Lilly & Co., Indianapolis, Ind.
[2] " " " S. E. Massengill Co., Bristol, Tenn.
[3] " " " The Squibb Institute for Medical Research, New Brunswick, N. J.
[4] " " " Parke-Davis & Co., Detroit, Mich.
[5] " " " Dow Chemical Co., Midland, Mich.
[6] " " " Abbott Laboratories, North Chicago, Ill.

group of animals given benzoic acid were considered the control group. The reasons for this were that the number of animals corresponded more closely with those in the other experimental groups and the animals were treated similarly in that they received an intraperitoneal injection of a potentially irritating solution which in each case contained an equivalent amount of sodium and was of identical pH. *Monohydroxy derivatives of benzoic acid.* The animals given o-hydroxybenzoic (salicylic) acid had highly significant elevations of plasma 17-OHCS concentrations. In contrast, the groups given m- or p-hydroxybenzoic acid did not have significant elevations of 17-OHCS concentrations at the 4 hour interval. *Ether derivatives of benzoic acid.* The o-substituted methoxy and ethoxy derivatives likewise were active in producing elevated 17-OHCS concentrations, the latter appearing perhaps to be more active than either the o-methoxy compound or salicylic acid. The elevations of plasma 17-OHCS produced by the m- and p-methoxy derivatives are of questionable significance because of wide variations in values among the animals tested; however, no significant effect was observed with p-ethoxybenzoic acid. On the other hand, 3,4,5-trimethoxybenzoic acid produced highly significant elevations of plasma 17-OHCS concentrations, possibly greater than those produced by salicylic acid ($p = .02$). *Amino derivatives of benzoic acid.* Two mono-amino benzoic acids, the o- and p- derivatives, were studied. Both of these compounds produced steroid elevations of borderline significance ($.02 < p < .05$ vs. benzoic acid). *Dihydroxy derivatives of benzoic acid.* Among the dihydroxybenzoic acids, the 2,3; 2,5 and 2,6-substituted derivatives resulted in highly significant plasma 17-OHCS elevations. Elevations of borderline significance ($.02 < p < .05$ vs. benzoic acid) occurred with the 2,4 and 3,5-substituted compounds. Insufficient data are available for the 3,4-substituted compound to permit conclusions. *Mixed derivatives of benzoic acid.* Derivatives of o-hydroxybenzoic (salicylic) acid with substitution of a methyl group at the 3 or 4 position or a methoxy group at the 3 or 5 position produced elevations of plasma 17-OHCS concentrations which were highly significant. With the small numbers of animals studied there appeared to be no significant difference between p-aminosalicylic acid and benzoic acid in their effects on 17-OHCS concentrations.

The greatest mean elevation of plasma 17-OHCS concentrations in any group of animals occurred in that group given acetylsalicylic acid (aspirin), the response being significantly greater than that obtained with salicylic acid. Salicyluric acid exerted no effect on plasma 17-OHCS concentrations. *Time relationship of steroid response to monohydroxybenzoic acids.* Table II concerns plasma 17-OHCS concentrations at varying intervals following treatment with the monohydroxybenzoic acids. Animals treated with o-hydroxybenzoic (salicylic) acid had no significant elevation of plasma 17-OHCS concentrations at 2 hours, but had highly significant elevations at 4 hours. Those treated with m-hydroxybenzoic acid showed a suggestive ($.02 < p < .05$ vs. benzoic acid) elevation of 17-OHCS concentrations at 2 hours. p-Hydroxybenzoic acid did not significantly affect 17-OHCS concentrations at any of the 3 sampling intervals (1, 2 or 4 hours).

TABLE II. Plasma 17-OHCS in Guinea Pigs at Varying Intervals following Treatment with Mono-hydroxy-Benzoic Acids. (Doses, 200 mg/kg by intraperitoneal injection.)

Compound*	Sampling interval 1-hr	2-hr	4-hr
o-Hydroxybenzoic (salicylic) acid		44 ± 10.9 (3)	81 ± 5.6 (5)
m-Hydroxybenzoic acid	63 ± 7.3† (3)	71 ± 8.5 (4)	58 ± 9.3 (4)
p-Hydroxybenzoic acid	49 ± 8.8 (4)	58 ± 6.6 (2)	57 ± 3.0 (5)

* Each administered as the sodium salt.
† Mean ± S.E.
No. in parentheses = No. of determinations.

Discussion. Equimolar doses of each of the compounds studied were administered in order to permit estimations of relative potency of these compounds in producing elevations of circulating 17-OHCS concentrations. However, certain variables preclude strict comparisons. Substantial differences may exist, among the compounds tested, in absorbability from the peritoneal cavity. The sampling interval of 4 hours was chosen because the maximum steroid response to salicylate was found to occur at that time(1). However, maxima for the other benzoic acid derivatives may not have coincided with the time of sampling used in these studies. Moreover, although the dose of each compound was equivalent on a molar basis there was wide variance in the ratio of administered to toxic dose. A non-specific toxic effect therefore is more likely with some of these substances than with others. None of the compounds studied was lethal in the dose employed. The majority of the compounds used in the present investigation have been studied only to a limited degree, or not at all, with respect to antiphlogistic potency. Moreover, where such data are available, categorical conclusions as to whether or not the particular compound has antiphlogistic properties cannot be drawn in most instances since results have differed from one type of experiment to another. Therefore, any comparison of this effect with influence on 17-OHCS concentrations necessarily must be incomplete and will be attempted only insofar as available data permit.

Among the monohydroxy derivatives of

benzoic acid, only the o-substituted compound (salicylic acid) exerted a pronounced effect on 17-OHCS concentrations at the 4 hour post-injection interval. Blanchard, *et al.*(5) and Cronheim, *et al.*(6) found the m-compound to have no effect on adrenal ascorbic acid, but others(7-10) have found that it produces significant depletion. Smith(9) reported significant adrenal ascorbic acid depletion with both m- and p-hydroxybenzoate. However, the doses employed (330 and 400 mg/kg, respectively) were much higher than those used in the present study and produced less depletion of adrenal ascorbic acid than did comparable doses of salicylate or even benzoate. The author attributed the differences in response to the lower circulating concentrations of the m- and p-hydroxybenzoates. He found that the peak plasma concentration of p-hydroxybenzoate occurred 30 minutes after intraperitoneal injection; none could be detected 1½ hours later. The greatest depletion of adrenal ascorbic acid was noted after the former, and no significant depletion after the latter, interval. This finding was suggestive that the interval between injection and removal of the adrenals may have been too long in the experiments of other workers(5,6) who failed to demonstrate an effect of this compound on adrenal ascorbic acid content. The failure in the present study to demonstrate a significant effect on circulating 17-OHCS at a time when the salicylate effect is maximum (4 hours after injection) might be explained similarly. For this reason, further studies of animals treated with m- and p-hydroxybenzoic acid were made at one- and 2-hour intervals. In the case of p-hydroxybenzoic acid, no effect on 17-OHCS concentrations was demonstrable. With m-hydroxybenzoic acid there was a mean 17-OHCS concentration at 2 hours which was elevated by comparison with benzoic acid-treated ($.02<p<.05$), or untreated ($p<.01$), animals; no significant effect was demonstrable at 4 hours following injection. Of the 3 monohydroxy derivatives of benzoic acid, only o-hydroxybenzoic (salicylic) acid has been reported to have antirheumatic properties(11) and to inhibit the development of "anaphylactic arthritis"(12).

The effects of certain other derivatives of benzoic acid on circulating 17-OHCS concentrations appear not to parallel their effects on adrenal ascorbic acid concentrations. Although o-methoxy and o-ethoxybenzoic acid both caused definite elevations of 17-OHCS concentrations, these compounds have been found not to deplete adrenal ascorbic acid (6,7). Plasma 17-OHCS concentrations following m- or p-methoxybenzoic acid were so variable that although the mean 17-OHCS values in these groups were comparable with those of salicylate-treated animals, these elevations were not statistically significant with the small numbers of animals used. Of these compounds, only p-methoxybenzoic acid has been found to deplete adrenal ascorbic acid significantly(7). p-Ethoxybenzoate has no effect either on 17-OHCS concentrations or adrenal ascorbic acid(6).

Administration of the o- and p-amino derivatives of benzoic acid resulted in mean 17-OHCS concentrations which were intermediate between those produced by benzoic acid and salicylate. p-Aminobenzoic acid has been reported to have no effect on adrenal ascorbic acid concentration(6) and to have no(13), or suggestive(14), antirheumatic properties. Neither compound was reported to inhibit anaphylaxis(15).

The addition of a second hydroxyl or an ether group to the salicylate structure appeared, in certain instances, to enhance the effect on circulating 17-OHCS. Thus, treatment with 2,3-dihydroxybenzoic acid resulted in suggestively, ($.02<p<.05$), and 2-hydroxy, 3-methoxy- or 2,6-dihydroxy-benzoic acid in definitely ($p<.01$), greater elevations of 17-OHCS concentrations than did salicylic acid. 2,6-Dihydroxy benzoate (γ-resorcylate) has considerably greater antirheumatic potency than salicylate(16). Gentisic acid had essentially the same magnitude of effect on 17-OHCS concentrations as salicylic acid and has been reported to have comparable antirheumatic potency, despite observations that it produces a somewhat lesser depletion of adrenal ascorbic acid(6,7,10).

A somewhat surprising finding was the significantly greater effect on circulating 17-OHCS concentrations of aspirin than of salicylic acid when the 2 compounds were given in equimolar amounts. Indeed, aspirin produced the greatest effect on 17-OHCS concentrations of any of the compounds tested. The increment in steroid response to aspirin over salicylic acid is proportional to the increment in milligram dose, suggesting that the 2 compounds may have similar potency when administered in equal quantities.

Salicyluric acid, the principal metabolite of salicylate, exerted no effect on 17-OHCS concentrations. This substance has been reported to cause a questionable depletion ($p = .02$) of adrenal ascorbic acid(6) but no inhibitory effect on "anaphylactic arthritis" (12).

Since relatively few of the compounds investigated in the present study have been evaluated with regard to antirheumatic potency, it is difficult to assess the relationship of this property to ability to produce elevated circulating corticosteroid concentrations. However, insofar as available data permit such comparison, these two effects appear to correlate well. On the other hand, there appear to be numerous discrepancies between adrenal ascorbic acid-depleting ability of these drugs and either their antirheumatic effects or their influence on plasma 17-OHCS concentrations.

Summary. 1. Twenty-five compounds representing structural derivatives of benzoic acid were studied with regard to their effects on circulating 17-hydroxycorticosteroid (17-OHCS) concentrations in guinea pigs. 2. Benzoic acid had no effect on these concentrations. 3. Among the monohydroxybenzoic acids, the o- and m-substituted derivatives produced elevated plasma 17-OHCS concentrations. The latter compound exerted its effect earlier following administration than did salicylate. p-Hydroxybenzoate had no significant effect. 4. Among the mono-ether derivatives of benzoic acid only the o-substituted compounds produced significant elevations of plasma 17-OHCS concentrations. Significant elevations were produced by 3,4,5-trimethoxybenzoic acid. 5. Both o- and p-aminobenzoic acid produced steroid elevations of borderline significance. 6. The addition to the salicylate structure of a second hydroxyl group in the 3 or 6 position or of an ether group in the 3 position appeared to augment the effect on 17-OHCS concentrations. 7. Aspirin exerted the greatest effect on plasma 17-OHCS concentrations of any compound tested. 8. The salicylate metabolite, salicyluric acid, had no effect. 9. A comparison of the influence of these compounds on 17-OHCS concentrations with antirheumatic and anti-inflammatory properties and with effects on adrenal ascorbic acid concentrations, as reported by others, was attempted.

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1. Done, A. K., Ely, R. S., and Kelley, V. C., *J. Pediat.*, 1954, v44, 153.

2. Good, T. A., Done, A. K., Ely, R. S., and Kelley, V. C., *Metabolism*, in press.

3. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol.*, 1952, v12, 519.

4. Eik-Nes, K., Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.*, 1953, v13, 1280.

5. Blanchard, K. C., Dearborn, E. H., Maren, T. H., and Marshall, E. K., Jr., *Bull. Johns Hopkins Hosp.*, 1950, v86, 83.

6. Cronheim, G., King, J. S., Jr., and Hyder, N., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 51.

7. Lowenthal, J., Jaques, L. B., *J. Pharmacol. and Exp. Therap.*, 1953, v107, 172.

8. Van Cauwenberge, H., and Roskam, J., *Compt. rend. Soc. Biol.*, 1953, v147, 1118.

9. Smith, M. J. H., *Lancet*, 1952, v1, 991.

10. Feeney, G. C., Carlo, P., and Smith, P. K., *J. Pharmacol. and Exp. Therap.*, 1955, v114, 299.

11. Stockman, R., *Rheumatism and Arthritis*, 1920, Edinburgh.

12. Ungar, G., Damgaard, E., and Hummel, F. P., *Am. J. Physiol.*, 1952, v171, 545.

13. Dry, T. J., Butt, H. R., and Sheifley, C. H., *Proc. Staff Meet. Mayo Clin.*, 1946, v21, 497.

14. Rosenblum, H., and Fraser, L. E., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 178.

15. Lepper, M. H., Caldwell, E. R., Jr., Smith, P. K., and Miller, B. F., *ibid.*, 1950, v74, 254.

16. Reid, J., Watson, R. D., Cochran, J. B., and Sproull, D. H., *Brit. Med. J.*, 1951, v2, 321.

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Errors in Plasma Volume Measurement from Adsorption Losses of Albumin-I^{131}.* (22737)

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Recently, when using albumin-I^{131} to measure plasma volume, we obtained unexpectedly low results. Adsorption losses of the albumin-I^{131} to the walls of containers offered a possible explanation, and experiments showed this to occur. The degree of error introduced in plasma volume measurement by this adsorption and methods of preventing it have been studied.

Methods. Various samples of a commercial preparation of human albumin-I^{131},[‡] containing 50 to 140 μC I^{131} per mg albumin when received, were used. Ninety-six to 98% of the radioactivity was protein-bound as shown by precipitation with 5% trichloroacetic acid. When received the material containing 1 mC was diluted in 30 ml sterile 0.9% NaCl. This stock solution was stored in the refrigerator and discarded after 3 to 4 weeks. Radioactivity was measured in capped vials in a well scintillation counter. All glassware was cleaned in concentrated chromic acid-sulfuric acid cleaning mixture (100 g K dichromate in 200 ml H_2O, treated with 1000 ml conc. H_2SO_4) for from 0.5 to 24 hours, then rinsed repeatedly with hot water, cold water and distilled water. Of the many solvents tested only chromic acid-sulfuric acid completely removed the adsorbed radioactivity from glass in a few minutes at 25°C. Hence this mixture was used for "extracting" the bound radioactivity. The loss by adsorption to pipettes and containers made special precautions necessary for the accurate preparation and assay of dilute solutions of albumin-I^{131}. Losses proved negligible in a micrometer syringe filled with a solution containing 200 or more μg protein/ml, and more dilute solutions were therefore prepared by delivering a minute volume of a relatively concentrated solution from this syringe into the solvent. To prevent losses in 1 ml transfer pipettes, these were first filled and emptied thrice with a 1 mg/ml solution of plasma albumin or dilute human plasma. Then air was sucked through them till the lower 3 inches of the pipette was almost dry. After this treatment, at most 2% of the radioactive contents was adsorbed.

Results. Table I summarizes the results of a typical experiment in which 27 μg of albumin-I^{131} were transferred to 4 pairs of 50 ml and 4 pairs of 250 ml flasks. To each pair was added one of the 4 solvents—distilled water, 0.9% NaCl, 0.1 N NaOH and 0.9% NaCl containing 1 ml human plasma/100 ml. The flasks were mixed by repeated inversion initially and before removing the samples. It is seen that counts are lost from water and sodium chloride solution and that these losses increase with storage at low temperature overnight and are proportionately greater from the more dilute solutions. Most of the lost counts are regained within a few minutes after adding NaOH. Counts are not lost from stored solutions which contain the carrier protein. A number of similar experiments showed: a) that KI in 1% concentration or less did not prevent loss of counts from water solutions; b) 3 and 5% NaCl, 1 and 3% $NaHCO_3$, and 3% Na Acetate did not prevent loss of counts; c) irregular small losses of counts occurred from 0.1 N NaOH and 1% Na_2CO_3 solutions; d) the 1% detergent solutions used by Sear *et al.*(6)[§] completely blocked the loss of counts for several days at 4°C; e) addition of sufficient human serum albumin or human plasma, with or without KI, blocked the loss of counts from 0.9%

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[†] At present on duty with the U. S. Air Force (M.C.).

[‡] From Abbott Laboratories, N. Chicago, Ill.

[§] One percent Alconox cleaning solution.

TABLE I. Loss of Counts from Albumin-I^{131} Solutions in Volumetric Flasks.

	Distilled H_2O	.9% NaCl	.1 N NaOH	Carrier protein in .9% NaCl
A. 27 μg Albumin-I^{131} in 50 ml of solvent				
Counts/min./ml				
After 90 min. at 25°C	18240 (85%)	19160 (89 %)	21740 (101 %)	21560 (100 %)
After 20 hr at 4°C	13700 (64%)	17100 (80 %)	21000 (97.5%)	21360 (99 %)
B. 27 μg Albumin-I^{131} in 250 ml of solvent				
Counts/min./ml				
After 90 min. at 25°C	3190 (75%)	3760 (88.5%)	4390 (103 %)	4250 (100 %)
After 20 hr at 4°C	1960 (46%)	3050 (72 %)	4160 (98 %)	4350 (102.5%)
After adding 300 mg NaOH	4160 (98%)	4040 (95 %)		

NaCl completely for as long as 2 weeks at 4°C.

Figure 1 shows the results of 2 of a number of experiments designed to measure the adsorption of protein to glass from 0.9% NaCl solution at room temperature. In Exp. A, 3 ml protein solution was exposed to the inner surface of 10 × 75 mm Pyrex test tubes capped with Parafilm and mixed by inversion initially and at 10 minute intervals thereafter; in Exp. B, 15 ml protein solution was exposed to the inner surface of 15 × 125 mm Pyrex test tubes with mixing only initially. At concentrations of protein of the order of 1 μg/ml or less, 10% to 50% of the protein is adsorbed to the walls of the test tubes. As the protein concentration increases the proportion of the total adsorbed decreases, until at concentrations of between 10 and 30 μg/ml, only 2 to 5% of the total is adsorbed. In Fig. 2 the μg protein adsorbed per cm² glass in Experiments A and B are plotted against the *final* concentrations of protein in solution. (The *final* concentrations were obtained by subtracting the quantity adsorbed from the quantity added initially to the test tube). The quantity of protein adsorbed by unit area of surface increases over the whole range of concentrations studied, but most rapidly over the range from 0.01 to 5 μg/ml (Fig. 2 insert). A few experiments showed that when 3 ml solution of 2 μg albumin-I^{131}/ml saline is mixed by repeated inversion in 10 × 75 mm test tubes, one half the equilibrium value is adsorbed in from 1 to 5 minutes and equilibrium appears to be reached in from 5 to 20 minutes. Albumin-I^{131} adsorbs to surfaces other than glass. It adsorbs vigorously to polyethylene, as noted by Sear, Allen and Gregersen(6). We have observed that albumin-I^{131} also adsorbs to celluloid and other transparent plastics, to glass tubes coated with silicone and histological paraffin, and to stainless steel and rubber.

Discussion. Different samples of human albumin-I^{131} show some variation in adsorption but in general the adsorption isotherms

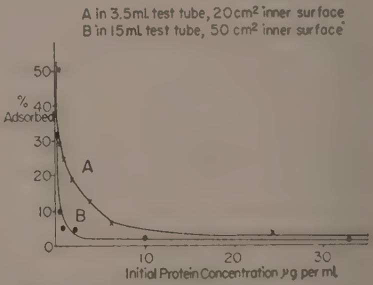

FIG. 1. % of total albumin-I^{131} adsorbed in 1 hr plotted against initial conc. (μg/ml).

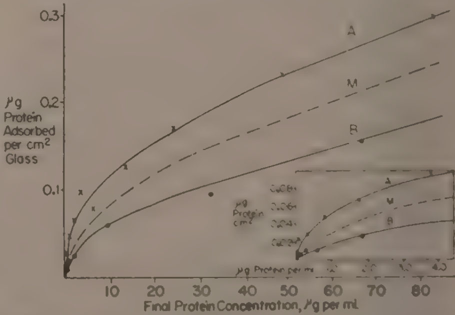

FIG. 2. Relation between protein conc. and quantity of protein adsorbed to glass.

lie in the area bounded by curves A and B, Fig. 2. We find also that rabbit albumin-I^{131} and human gamma globulin-I^{131} adsorb similarly. Recently Bull(2) has reported quantities of unlabelled bovine albumin adsorbed to glass from buffer of pH 4.66 that would fit well with a projection of curve A, Fig. 2. The errors due to adsorption in a plasma volume measurement depend on the fraction of the total albumin-I^{131} adsorbed to the glassware. This depends on the solvent, protein concentration, area of surface, time of exposure to surface, temperature and the effectiveness of mixing in the solution. At room temperature with 0.9% NaCl as solvent the approximate errors resulting from adsorption can be calculated from Curve M, the mean of curves A and B, Fig. 2, provided that the surface area of the glassware and the protein concentrations of the solutions are known. Calculations show that the surface volume relationships are of great importance. Thus, volumetric glassware with a surface of less than 1 cm^2 per ml contained solution adsorbs less than 1% of the contained protein at concentrations of 10 μg/ml, and less than 5% at concentrations of 1 μg/ml, whereas, glassware with a surface of 20 cm^2 per ml may adsorb 15% of the contained protein at 10 μg protein/ml, and 35% at 1 μg/ml. Examples of the former glassware are volumetric flasks of 100 ml capacity or greater, and of the latter, 1 ml Tuberculin syringes and straight 1 ml pipettes. (Pipettes would be a very grave source of error were it not that they are filled and emptied rapidly, and, since albumin diffuses slowly, time is insufficient for equilibrium to be reached. Experiment shows that with each filling a few per cent of the contents is adsorbed until the surface becomes saturated.) The calculated percentages of protein that become adsorbed during a plasma volume measurement, in which 10 ml of 0.9% NaCl solution of albumin-I^{131} containing 10 to 40 μg protein/ml are injected, and the radioactivity in 1 ml of plasma separated from the blood samples is compared with that of 1 ml of a 1 in 100 dilution of the injection solution are: In the syringe, 3 to 6; in the glassware containing the separated plasma, nil; in the glassware used for the standards (a) 100 ml volumetric flask, 8 to 10; (b) two 1 ml transfer pipettes, 2 to 3 each. Since losses in the syringe lead to an overestimate whereas those occurring during the preparation of the standards lead to an underestimate of plasma volume, these errors partially cancel out. As a result, plasma volume is underestimated by 8 to 12% depending on the protein concentration of the injected solution. In unfavorable circumstances such as storage of standards in the cold and preparation of standards with distilled water, greater errors would arise. Schultz *et al.*(5) who measured radio activity with G-M counters, added plasma to their standards to equalize adsorption losses of B rays; Sear *et al.*(6) prepared their standards in 1% detergent. These two groups of workers showed good agreement between plasma volume measurements made in man and dog with T1824 and albumin-I^{131}. Other workers (*e.g.*1,7) have failed to find such good agreement. Recently, technics using scintillation counters have been described(3) in which losses by adsorption would be anticipated; Gamble *et al.*(4) have already noted such losses. It is clear that to make precise measurements these losses must be avoided. This may be achieved as follows: When the albumin-I^{131} is prepared for injection at least 1 mg carrier protein per ml solution is added. For carrier protein either the patient's plasma or a preparation of human plasma albumin is used. To make the standards a measured volume of the solution prepared for injection is diluted in a volumetric flask, either with 0.9% NaCl containing 0.5 mg plasma protein/ml, or with 1% detergent solution.

Summary. Adsorption losses of albumin-I^{131} to volumetric glassware may be sufficient to cause significant underestimates of plasma volume. Some factors affecting these losses are examined and methods of minimizing their effects are described.

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1. Aust, J. B., Chou, N. S., Marvin, J. F., Brackney, E. L., and Moore, G. E., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 514.

2. Bull, H. B., *Biochim. et Biophys., Acta,* 1956, v19, 464.

3. Erickson, J. R., McCormick, J. B., and Seed,

L., *Science*, 1953, v118, 595.

4. Gamble, J. L., Jr., Klement, A. W., Jr., and Rogers, E. B., PROC. SOC. EXP. BIOL. AND MED., 1953, v85, 172.

5. Schultz, A. L., Hammarsten, J. F., Heller, B. I., and Ebert, R. V., *J. Clin. Invest.*, 1953, v32, 107.

6. Sear, S., Allen, T. H., and Gregersen, M. I., *Am. J. Physiol.*, 1953, v175, 240.

7. Zipf, R. E., Webber, J. M., and Grove, G. R., *J. Lab. Clin. Med.*, 1955, v45, 800.

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Erythropoietic Stimulating Effects of Plasma Extracts from Anemic Human Subjects. (22738)

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It is now a well documented finding that various types of anoxia evoke the production, in laboratory animals, of circulatory factors capable of stimulating erythropoiesis(1-11). These observations raise the question as to whether such activity is also demonstrable in the blood of patients with various forms of anemia. Few studies have attempted to assess the erythropoietic (EP) properties of body fluids of human subjects. Some of the experiments have been performed on umbilical cord blood of normal newborn babies and have indicated EP activity in such material when tested in rodents and man(12-16). EP activity is also stated to be present in the serum of a man sojourning at altitude for 8 days (12), in the plasma of patients with chronic heart disease(1), in human subjects with anemia following hemorrhage(17) and in infants with erythroblastosis fetalis(16). Plasma from subjects with congenital spherocytosis or pernicious anemia in reticulocyte crisis was ineffective(18). These studies with human material fail to evaluate the response of the blood-forming organs in the test animals. The criteria used by the investigators, namely red cell (RBC) counts and/or reticulocyte response, cannot always be depended upon as reflecting the degree of EP stimulation(19,20).

The present studies are concerned with the effects exerted by extracts of plasma and one of urine from patients with certain types of anemia. Since previous experiments(5-8,10) indicate that more of the EP factor is recoverable in hemolytic than in other types of experimentally induced anemias, possibly because the hemolytic varieties are usually more severe, it was felt that an examination of the effects of body fluids secured from patients with Cooley's and sickle cell anemia might be rewarding.

Materials and methods. Plasma was obtained from 7 authenticated cases of Cooley's anemia (6 children and 1 adult) and 2 patients with sickle cell anemia. One case of chronic hypoplastic anemia in a child is also included. Four normal subjects served as donors for control plasma. A 24 hour sample of urine from one of the Cooley's subjects was also tested. In Table I are indicated the hemoglobin concentration, reticulocyte percentage and nucleated RBC numbers in the peripheral blood of each of these individuals at the time of blood withdrawal.

Because of the effectiveness of an acidification-boiling procedure in revealing the EP factor in the blood and urine of anoxic rodents (5-7,10,11,21), it was decided to utilize this extraction technic for the plasmas of the anemic patients. Blood collected from each of the patients was heparinized (10 mg/50 ml blood). The plasmas were acidified to pH 5.5 with 1N HCl and boiled for 10 minutes. Following filtration, the coagulated residues were discarded and the filtrates neu-

TABLE I. Effects of Plasma from Anemic Patients upon Erythropoiesis in the Intact Rat.

Peripheral blood values of patient					Effects in recipient rats (means ± S.E.)					
Age, yr		Hb (g %)	Retics (%)	Nuc. RBC (per 100 WBC)		RBC (mill/mm³)	Hb (g %)	Hemat. (%)	Retics (%)	Marrow nuc. RBC (%)
I. Cooley's anemia										
5	♂	5.5	2.1	4	B*	8.82 ± .50	17.57 ± .87	46.6 ± 2.7	1.1 ± .30	
					A*	9.77 ± .74	18.03 ± .98	54.4 ± 2.0	6.7 ± .55	46.8 ± .2
10	♂	7.5	.6	3	B	8.90 ± .53	16.51 ± .15	47.5 ± .50	1.2 ± .35	
					A	10.30 ± .17	17.40 ± .21	49.0 ± 1.0	1.4 ± .40	45.5 ± 7.5
5	♀	5.0	1.1	0	B	8.05 ± .90	16.56 ± .90	47.7 ± .90	2.2 ± .30	
					A	10.24 ± .21	19.26 ± .43	59.0 ± 1.1	11.3 ±1.40	51.5 ± 3.5
5	♀	7.0	2.5	4	B	8.11 ± .57	17.75 ± .28	49.6 ± .40	1.8 ± .50	
					A	9.17 ± .23	18.52 ± .35	52.5 ± 2.50	1.9 ± .40	32.5 ±17.5
4	♀	6.0	3.5	13	B	8.41 ± .90	18.51 ± .35	52.8 ± 2.30	1.5 ± .60	
					A	9.85 ± .25	18.94 ± .08	60.5 ± 2.53	5.2 ± .70	44.5 ± 2.5
12	♂	8.0	7.2	240	B	8.79 ± .24	18.45 ± .56	51.1 ± .10	2.1 ± .50	
					A	9.06 ± .68	18.93 ± .76	57.2 ± 1.80	3.0 ±1.10	45.5 ± 2.5
32	♀	9.5	6.0	1	B	8.42 ± .30	17.56 ± .65	49.6 ± 1.90	2.5 ± .19	
					A	9.51 ± .22	18.80 ± .31	52.5 ± 1.10	4.7 ± .64	43.6 ± 1.2
II. Sickle cell anemia										
11	♂	8.0	6.4	0	B				1.8 ± .26	
					A				5.6 ± .56	52.3 ± 1.2
15	♂	7.5	1.0	2	B	8.94 ± .28	16.36 ± .15	49.0 ± 2.01	1.9 ± .38	
					A	9.13 ± .34	17.38 ± .47	51.6 ± 2.06	2.4 ± .06	28.0 ± 2.7
III. Hypoplastic anemia										
7	♂	5.0	0	0	B	9.34 ± .22	18.22 ± .80	51.5 ± 1.34	2.2 ± .15	
					A	9.54 ± .47	18.86 ± .24	52.3 ± .93	3.8 ± .59	34.3 ± 4.1
IV. Controls										
12	♂	12.5	1.0	0	B	8.44 ± .70	17.15 ±1.52	47.5 ± 1.11	2.6 ± .12	
					A	8.36 ± .36	15.93 ± .14	43.5 ± .95	3.6 ±1.23	32.0 ± 1.0
12	♂	13.0	.4	0	B	8.61 ± .29	16.76 ± .24	50.6 ± 1.36	1.7 ± .62	
					A	8.77 ± .54	16.78 ± .02	49.8 ± 1.24	1.2 ± .03	27.5 ± 3.5
5	♂	12.0	.2	0	B	9.04 ± .09	18.27 ± .39	49.6 ± 2.17	2.0 ± .85	
					A	8.40 ± .28	17.26 ± .90	49.1 ± 1.19	1.8 ± .13	27.0 ± 3.0
6	♂	10.0	.4	0	B	8.75 ± .16	18.20 ± .15	48.8 ± .36	1.9 ± .21	
					A	8.65 ± .04	16.70 ± .34	46.6 ± .65	1.4 ± .15	33.5 ± 1.5
V. Cooley's anemia Urine extract										
(See above)		5.5	2.1	4	B				1.5 ± .23	
					A				3.6 ± .61	45.2 ± 1.1

* B—Before treatment; A—After 5 injections.

tralized with 1N NaOH. In some cases saline was added during or after the boiling procedure in order to achieve a volume close to that of the original plasma. A similar acidification and boiling technique was used for the specimen of urine obtained from one of the patients with Cooley's anemia.

In view of the relatively small amounts of blood available, and to avoid the obviously inadequate procedure of pooling the plasmas of the different patients, no more than 2 to 3 recipient rats (young adult females of a modified Long-Evans strain) could be used in assessing EP activity in the plasma extract of each subject. Each rat received subcutaneous injections, daily for 5 days, of 1.8 to 3.0 ml of filtrate, equivalent in all cases to approximately 3.0 ml of original plasma. Five rats were employed to test the EP properties of the extract prepared from the urine of one of the Cooley's patients. Each rat received daily subcutaneous injections for 5 days of 1.5 ml of the urine extract, equivalent to 3.0 ml of the original urine. All rats were killed on the 6th day for erythrocytic studies of the peripheral blood and bone marrow as described previously(7,22).

Results. It will be observed from Table I that in all cases, increases in the RBC count, hemoglobin and hematocrit values occurred in the rats receiving the plasma filtrates of patients with Cooley's anemia. Marked reticulocytosis was evident in Cases 1, 3 and 5, and strong stimulation of marrow erythropoiesis was noted with all extracts except that obtained from Case 4. With the latter extract, one marrow possessed 50% but the other only 15% nucleated RBC. Previous studies(11) have shown that marrows from 40 untreated rats of the same strain and sex possess 35.1 ± 1.9% nucleated RBC. A similar degree of EP stimulating activity, as judged by the reticulocyte and marrow response (only parameters examined), was revealed in the extract of plasma from one of the 2 cases (Case 1) of sickle cell anemia. The plasma extract from the second patient proved ineffective. None of the extracts obtained from the control donor subjects stimulated erythropoiesis in the recipient rats.

The extract of urine obtained from a child with Cooley's anemia (Case 1) also stimulated erythropoiesis, as noted from the significant reticulocytosis and the increased percentages of nucleated RBC in the marrows of the recipient rats (Table I). Unfortunately, in this case, the other peripheral erythrocytic parameters of the test rats were not ascertained.

The case of chronic hypoplastic anemia examined was a boy possessing this condition since birth. His RBC count, at the time of obtaining the peripheral blood sample was 1.8 mill/mm^3 and sternal biopsy indicated only 2% nucleated RBC within his bone marrow. Despite the intensity of anemia in this subject, no EP activity in the plasma extract was demonstrable in recipient rats (Table I).

Discussion. It seems clear that considerable EP stimulating activity exists in the blood of patients with Cooley's anemia. Activity was also present in the plasma of one of 2 subjects with sickle cell anemia. The EP factor detected here is heat stable and can be recovered in boiled filtrates of the plasma and urine. In these respects, it resembles the material present in the plasma of rabbits and rats subjected to hemolytic or hemorrhagic anemia(5,6,10,21). The activity of human circulating "erythropoietin" as seen in some of the patients (*e.g.* Cooley case 3), is actually greater than that detectable in any of the bled or phenylhydrazine-treated animals examined by us up to date, considering that the material of human origin was administered for only 5 days. It would thus seem that either human "erythropoietin" is somewhat different in chemical structure from the animal type or, what is more likely, it is the same substance but exists in larger concentration in the blood of the anemic patients studied. It was of interest that the extract of urine from one of the patients with Cooley's anemia possessed EP properties. Considering that the urine was concentrated to no more than 50% of its original volume, the reticulocyte and marrow response in the recipient rats represent a considerable degree of EP activity. Others(21) have detected EP activity in extracts of urine from rabbits subjected to bleedings. It is also apparent from the data that no correlation can be drawn between severity of the anemia in the patient at the time of blood withdrawal and intensity of the EP response to the plasma extracts in the recipient rats. Nor can it be related to the duration of the condition in the patient. Evidently the quantity of "erythropoietin" circulating in the blood is a variable one, dependent undoubtedly not only on the rate of production of the factor but also upon its rate of utilization or destruction. Any of these processes could conceivably change from time to time.

The results indicate that erythromicrocytosis, which is sometimes characteristic of the response to the plasma EP factor(10,11) was not apparent in the rats receiving the plasma extracts from the Cooley cases 1, 3, 5 and 6 (*i.e.* the percentage increase in the hematocrit values matched the percentage rise in RBC numbers). In most instances, hemoglobin production was not stimulated to the same extent as RBC proliferation, thus resulting in a lowered corpuscular hemoglobin content.

Since the blood of the subjects with Cooley's anemia displays strong EP stimulating qualities and their marrows reveal marked erythroid hyperplasia, it would seem unlikely that a contributory factor to this condition is a reduction in the production or utilization of the EP factor. Undoubtedly, the more basic etiology centers about the abnormal hemoglobin and hemolytic mechanisms characteristic of these disease states(23-25). A significant finding was the absence of EP activity in the plasma extract of the patient with severe chronic hypoplastic anemia. It is possible that there is an impaired capacity of such subjects to elaborate the EP principle. Should this prove to be the case in more detailed studies of hypoplastic anemia, the administration to these subjects of purified "erythropoietin," preferably that obtained from human sources, would constitute a justifiable therapeutic measure.

Summary. Boiled filtrates of acidified plasma obtained from 7 patients with Cooley's anemia and one of 2 patients with sickle cell anemia stimulated erythropoiesis in intact rats. A similarly prepared extract of urine from one of the Cooley's subjects was also active. Plasma filtrates from normal subjects and one patient with chronic hypoplastic anemia were inactive. The implications of these findings are discussed.

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1. Bonsdorff, E., and Jalavisto, E., *Acta physiol. Scandinav.*, 1948, v16, 150.

2. Gley, P., *Bull. Acad. Méd.*, 1952, v136, 521.

3. Tohá, J., Hodgson, G., and Weasson, E., *Acta hematol.*, 1953, v9, 1.

4. Erslev, A., *Blood*, 1953, v8, 349.

5. Gordon, A. S., Piliero, S. J., Kleinberg, W., and Freedman, H. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 255.

6. Borsook, H., Graybiel, A., Keighley, G., and Windsor, E., *Blood*, 1954, v9, 734.

7. Gordon, A. S., Piliero, S. J., Tannenbaum, M., and Siegel, C. D., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 246.

8. Gordon, A. S., Piliero, S. J., and Tannenbaum, M., *Am. J. Physiol.*, 1955, v181, 585.

9. Plzak, L. F., Fried, W., Jacobson, L., and Bethard, W., *J. Lab. and Clin. Med.*, 1955, v46, 671.

10. Linman, J. W., and Bethell, F. H., *Blood*, 1956, v11, 310.

11. Gordon, A. S., Piliero, S. J., Medici, P. T., Siegel, C. D., and Tannenbaum, M., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 598.

12. Loeschke, E., and Schwartzer, K., *Monatschr. f. Kinderh.*, 1939, v81, 25.

13. Döring, G. K., *Pflueg. Arch. f. d. ges. Physiol.*, 1948, v249, 631.

14. Klingelhöffer, K. O., *ibid.*, 1948, v250, 465.

15. Döring, G. K., and Loeschke, H. H., *ibid.*, 1949, v251, 220.

16. Seip, M., *Acta Paediat.*, 1955, v44, 507.

17. Oliva, G., Chiuinini, F., and Tramonta, C., *Acta med. Scandinav.*, 1949, v133, 27.

18. Seip, M., *ibid.*, suppl. 1953, v282, 1.

19. Gordon, A. S., and Charipper, H. A., *Ann. N. Y. Acad. Sci.*, 1947, v48, 615.

20. Gordon, A. S., *Rec. Prog. Horm. Res.*, 1954, v10, 339.

21. Hodgson, G., and Tohá, J., *Blood*, 1954, v9, 299.

22. Gordon, A. S., Piliero, S. J., and Landau, D., *Endocrinology*, 1951, v49, 497.

23. London, I. M., Shemin, D., West, R., and Rittenberg, D., *J. Biol. Chem.*, 1949, v179, 463.

24. Pauling, L., *Harvey Lect.*, Ser. 49, 1953-4, 216.

25. James, G. W., III, and Abbott, L. D., Jr., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 398.

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Effect of Route of Administration on Protective Action of Corticosterone and Cortisol Against Endotoxin. (22739)

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The protective effect of cortisone (11 dehydro, 17-OH corticosterone) on lethal action of bacterial endotoxin(1-5) is in contrast to the usual adverse effects of cortisone and cortisol (17-OH corticosterone) on resistance to infection(6). This protective effect has been demonstrated in mice, rats and rabbits using endotoxin from several bacterial sources and its demonstration has generally required that the cortisone be given before or simultaneously with endotoxin(1-5).

Cortisone and cortisol have similar adverse effects on resistance to infection and on antibody production, but corticosterone, which is the predominant corticosteroid secreted by rats and, under ordinary circumstances, by rabbits, is less active than its congeners in these respects(7,8). It was therefore of interest to compare the actions of corticosterone and cortisol with respect to their effects on the lethal action of endotoxin.

Materials and methods. Animals. White male rats of a stock strain derived from the Sprague-Dawley strain, and weighing 130-180 g, were used throughout. They were fed a stock laboratory diet and water *ad libitum*. *Endotoxin.* The endotoxin was prepared from 15 liter amounts of culture of *S. typhi* (Ty 2), grown according to the method of Formal and Baker(9).† After overnight incubation 30 ml of chloroform and 250 mg of crystalline trypsin were added and allowed to act for 24 hours. Two volumes of acetone were then added and the sediment collected by centrifugation. The sediment was suspended in one liter of distilled water, the large particles removed by centrifugation and the crude endotoxin was precipitated out by addition to 2 liters of 95% ethyl alcohol and 1 liter of ethyl ether. The sediment was collected by centrifugation, resuspended in one liter of distilled water and again centrifuged. The supernatant was added to 1500 ml of ethyl alcohol and 750 ml of ether. The sediment was collected and suspended in 750 ml ether. The sediment was again collected, suspended in 750 ml of saline, centrifuged and the supernatant was stored at —20°C in small aliquots. Saline dilutions of this crude endotoxin were made prior to each experiment. Preliminary titrations (Table I) showed that 1.2 ml of this material per kg of body weight, injected intracardially in a volume of 1 ml, killed approximately 80% of rats; this amount was used unless otherwise indicated. The syndrome that followed the injection of endotoxin was the characteristic one that has been described(10). Mortality was recorded after 24 hours. In most of the experiments, the control animals received saline and alcohol in a volume equal to that of injected corticosteroid. However, when it became clear that the subcutaneous and intraperitoneal administration of saline and alcohol did not influence the reaction of the rats to LD_{80} of endotoxin, the use of control injections of saline was discontinued. In all experiments involving intracardiac injection, however, the control animals received saline and alcohol by the same route. *Corticosteroids.* Cortisol and

TABLE I. Lethal Effect of Intracardiac Injection of Endotoxin in Rats.

Amt inj., ml/kg*	No. surviving/No. inj.	% mortality
.8	3/ 8	62
1.2	5/21	76
1.6	0/ 8	100

* Endotoxin diluted in saline so that inj. dose was contained in a final volume of one ml. Saline alone given intracardially in 1.0 and 2.0 ml volumes to a total of 18 rats was not apparently deleterious.

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† We are indebted to Dr. E. E. Baker for his generous aid in the preparation of the endotoxin.

corticosterone were used as the free alcohols.[‡] The crystals were suspended in 20% ethyl alcohol to the desired concentration and given in a volume of 1 ml by the subcutaneous or intraperitoneal route. Solutions for intracardiac use were made by dissolving the requisite amount of steroid in 95% ethyl alcohol and diluting with saline to a final concentration of 2.5%. Blood levels of cortisol were determined by a modification of the Porter-Silber method(11). Corticosterone concentrations were estimated by the method of Rosenman *et al.*(12). Although the methods are not completely specific the rates of disappeaarnce of the chromogens after the administration of the known steroid was taken to be a valid reflection of declining blood levels of the steroid. The data are presented uncorrected for endogenous chromogen. Blood for determinations of corticosteroid was obtained by exsanguination via cardiac puncture with the animals under light ether anesthesia. Blood from 2-4 rats was pooled in order to obtain sufficient serum for analysis and each point that is recorded represents a single pool. Corticotropin[§] was given intramuscularly in oil and beeswax, as previously described(8). Lipoadrenal extract[||] and desoxycorticosterone acetate were given in oil intramuscularly.

Results. 1. *Effect of route of administration on protective effect of cortisol and corticosterone* (Table II). Corticosterone or cortisol were given subcutaneously in 10 mg doses and the animals were challenged at various times thereafter by the intracardiac administration of LD_{80} doses of endotoxin. Cortisol was protective when given 24 hours in advance of the endotoxin whereas corticosterone was not. However, when the time interval between the administration of hormone and of endotoxin was shortened, corticosterone became increasingly protective. When the hormones were given at the same time as the endotoxin, corticosterone was almost as active as was cortisol.

TABLE II. Route of Administration and Protection by Cortisol and Corticosterone against Lethal Action of Endotoxin.

Route* and dose of hormone	Interval between hormone and endotoxin, hr	Cortisol	Corticosterone	Control
s.c., 10 mg	24	5/ 8[†]	0/ 8	1/ 8
	8	6/ 8	3/ 8	0/ 8
	2	8/ 8	6/ 8	3/ 8
	1	6/ 8	5/ 8	1/ 8
i.p., 10 mg	8	2/ 8	2/ 8	1/ 8
	.5	8/ 8	6/ 8	1/ 8
	−1	3/ 8	2/ 8	1/ 8
i.c., .5 mg	2	3/ 8	1/ 8	3/ 8
	1	5/16	2/16	5/16
	0	12/16	12/16	3/16

* s.c. = subcut.; i.p. = intraper.; i.c. = intracardiac.

[†] No. surviving/total No. inj.

After intraperitoneal administration, cortisol and corticosterone were not protective when the hormones were given 8 hours before endotoxin, but both were protective when given one-half hour before endotoxin. Intraperitoneal administration of steroid one hour after endotoxin led to markedly decreased protection as has been previously observed (5).

When cortisol or corticosterone were given simultaneously with endotoxin by the intracardiac route both steroids were active in protecting against endotoxin. The steroids were equally ineffective when given one hour or more before endotoxin by this route, indicating that the added steroid had probably disappeared from the blood in one hour.

II. *Rate of disappearance of steroid from blood of rats.* The differences in effectiveness of corticosterone and cortisol when given subcutaneously were evident when a relatively long interval existed between times of administration of steroid and endotoxin. To determine whether these differences were due in part to differences in rate of disappearance of the steroids from the blood stream, animals were given purified steroid by the intracardiac route. The rates of disappearance of corticosterone and of cortisol after 0.5 mg of each was given intracardially to rats were essentially the same (Fig. 1), if correction for the endogenous chromogen is made, suggesting

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[‡] Kindly supplied by Sharp & Dohme Division, Merck & Co.

[§] Kindly supplied by Armour Laboratories.

[||] Kindly supplied by Upjohn Co.

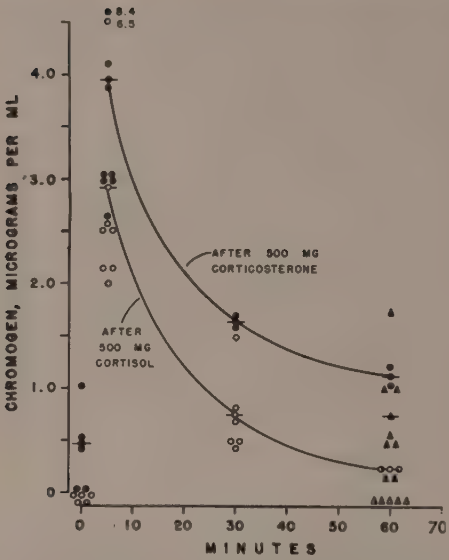

FIG. 1. Disappearance of cortisol and corticosterone from serum of rats after intracardiac administration. Solid symbols (●, ▲) refer to levels of 21-OH, 20 keto (corticosterone-like) chromogens, and open symbols (○, △) to 17,21 Di-OH, 20 keto (cortisol-like) chromogens. Triangles indicate blood levels of chromogens in control animals one hr after brief ether anesthesia had been given; circles show blood levels in resting control animals and following intracardiac inj. of either steroid.

that the differences in the effects of the 2 corticosteroids after subcutaneous administration were due largely to differences in their rates of mobilization from the local site of deposition.

III. *Protective effect of corticotropin and adrenal steroids against lethal action of endotoxin.* Corticotropin and lipoadrenal extract were protective against the lethal action of endotoxin (Table III), whereas desoxycorticosterone acetate was ineffective. Large doses of cortisol and corticosterone were, as previously mentioned, protective.

Discussion. The data demonstrate that corticosterone and cortisol were of similar effectiveness in protecting against the lethal action of endotoxin when the steroids were given directly into the blood stream at the time of administration of endotoxin. When, however, these steroids were given subcutaneously the protective effect of cortisol persisted for a longer period of time than did that of corticosterone. Since the rates of disappearance of these 2 steroids from the blood of the rats, after intracardiac administration, were similar, explanation of the differences in effects of these steroids must lie in the more rapid mobilization of corticosterone than of cortisol from the local site of deposition. Although differences in tissue mobilization or in rate of disappearance from the blood among steroids are well recognized(13,14), few experiments in which relative biologic activities of steroids are compared have taken these into account. It has been customary in experiments in this laboratory as well as in many others to regard corticosterone as having less biologic activity than cortisol or cortisone on the basis of the effect of widely spaced injections of hormone. It is apparent that many of the results of assays of the different biologic activities of these and related substances may require revision in the light of the findings herein reported.

That corticosterone and cortisol should prove to have similar activities when both are given directly into the blood stream is consistent with their close similarity in chemical structure and their occurrence singly or together as the predominant adrenocortical secretory products(15). Further study is required to determine if the reported differences between corticosterone and cortisol on inflammation and on resistance to infection and antibody formation(7,8) are related more to differences in mobilization of the steroid hormones from tissue sites of deposition than to major differences in their biologic activity when the steroids are presented to tissues at a constant rate.

The protective action of the corticosteroids

TABLE III. Protective Effect of Corticotropin and Adrenal Steroids on Lethal Action of Endotoxin.

Substance inj.	Dose*	No. surviving/No. inj.
Corticotropin	10 I.U.	7/8
DOCA	5 mg	2/8
Lipoadrenal	1 ml	5/8
Cortisol	10 mg	8/8
Corticosterone	10 "	6/8
Controls		3/8

* Test substance given intramusc. 48 hr, 24 hr, and 2 hr before inj. of endotoxin.

studied in these experiments is apparently related to increased concentrations of steroid in the blood during the time of exposure to endotoxin. Thus, one hour after intracardiac administration of corticosterone or cortisol, blood levels of corticosteroid had returned to normal values and the protective effect of the steroids was also absent.

The significance of the protective action of corticosteroid against endotoxin in terms of treatment is difficult to analyze. The protective effect was not demonstrable in dogs(16) or in guinea pigs(2,17); in animals in which it has been demonstrable, the effect was lost rapidly when steroid was given after endotoxin. In addition, Thomas and his coworkers(18) have shown that the prior administration of cortisone to certain rabbits prepared these animals for the development of generalized Shwartzman reactions after a single dose of endotoxin; the cortisone eliminated the need for the usual 2 doses of endotoxin. Only further study can determine whether corticosteroids influence endotoxic activity in established infections and to what extent corticosteroids alter the relationship between the adverse effect of corticosteroids on mechanisms of resistance to infection on the one hand, and the protective action of these steroids against endotoxin on the other.

Summary. 1. Both cortisol and corticosterone were effective in protecting rats against endotoxin when the steroid hormones were given intracardially at the time of administration of endotoxin. 2. Cortisol was more protective than corticosterone when both were given subcutaneously 24 hours before endotoxin; as the interval between the time of injection of steroid and the time of challenge with endotoxin was decreased, corticosterone was increasingly protective. 3. The rates of disappearance of corticosterone and cortisol from the blood of rats after intracardiac administration of steroid were similar. 4. The evidence indicates that corticosterone is more rapidly mobilized from local tissue sites of deposition than is cortisol and that the difference in the activity of these 2 substances after subcutaneous administration is due in large part to this difference. By inference, the many published differences in the biologic activities of cortisol and corticosterone may be subject to revision when control of this variable has been achieved. 5. Corticotropin and lipoadrenal extract were protective against endotoxin but desoxycorticosterone was not.

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1. Chedid, L., Boyer, F., and Saviard, M., *C. R. Acad. Sci.*, 1951, v233, 713.

2. Chedid, L., and Boyer, F., *Ann. Inst. Pasteur,* 1955, v88, 336.

3. Thomas, L., and Smith, R. T., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 810.

4. Spink, W. W., and Anderson, D., *J. Clin. Invest.*, 1954, v33, 540.

5. Geller, P., Merrill, E. R., and Jawetz, E., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 716.

6. Kass, E. H., and Finland, M., *Ann. Rev. Microbiol.*, 1953, v7, 361.

7. Kass, E. H., Lundgren, M. M., and Finland, M , *J. Exp. Med.*, 1954, v99, 89.

8. Kass, E. H., Kendrick, M. I., and Finland, M., *ibid.*, 1955, v102, 767.

9. Formal, S. B., and Baker, E. E., *J. Immunol.*, 1953, v70, 260.

10. Thomas, L, *Ann. Rev. Physiol.*, 1954, v16, 467.

11. Peterson, R. H., Wyngaarden, J. B., Guerra, D. L., Brodie, B. B., and Bunim, J. J., *J. Clin. Invest.*, 1955, v34, 1779.

12. Rosenman, R. H., St. George, S., Freed, S. C., and Smith, M. K., *ibid.*, 1955, v34, 1726.

13. Brown, H., Willardson, D. M., Samuels, L. T., and Tyler, F. H., *ibid.*, 1954, v33, 1524.

14. Ely, R. S., Done, A. K., and Kelley, V. C., PROC. SOC. EXP. BIOL. AND MED., 1954, v91, 503.

15. Hechter, O., and Pincus, G., *Physiol. Rev.*, 1954, v34, 459.

16. Ebert, R. V., Borden, C. W., Hall, W. H., and Gold, D., *Circ. Rev.*, 1955, v3, 378.

17. Reilly, J., Compagna, A., Tournier, P., and DuBuit, H., *Ann. Med.*, 1954, v55, 5.

18. Thomas, L., *Bull. New York Acad. Med.*, 1955, v31, 485.

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Studies on Thymus of the Mammal. IX. Histochemical Study of Irradiated Mouse Thymus.*† (22740)

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The increased amount of lipids in the thymuses of C57 mice after exposure to a single total-body dose of x-rays was described by Smith and Holst(1). Thymuses normally contain little or no lipid. However, they become heavily loaded with fat 24 and 36 hours after irradiation when the evidences of injury are greatest. They return to a normal condition in 7-9 days. The mediastinal lymph nodes do not undergo a similar severe reaction. In the present investigation attention was directed to the study of acid phosphatase. In normal thymuses there is little indication of activity of this enzyme but after irradiation the changes in the number and distribution of cells showing sites of acid phosphatase activity correlate in general with lipid deposition.

Material and methods. BAlb/c male mice, 30-40 days old from the Roscoe B. Jackson Memorial Laboratory were used. The thymus, spleen and mesenteric lymph node were removed from control animals and from mice 2, 4, 8, 12, 24, 36 hours and 3, 5, 7 and 9 days after exposure to irradiation. The mice killed at 12 (in part), 24, and 36 hours had received doses of x-ray of 410 r; the radiation factors were 250 K.V., no filter, 3 min., 50 cm TSD.‡ The animals sacrificed 2, 4, 8, 12 (in part) hours and 5, 7 and 9 days received 434-455 r, gamma rays from radioactive cobalt (42 cm aluminum filter).§ Gomori's method(2) was used for the demonstration of sites of activity of acid phosphatase. The thymus, spleen and mesenteric lymph node were removed from each animal, fixed in ice-cold acetone and embedded in paraffin together; sections were cut at 8 μ and mounted on the same slide. The paraffin was not removed from the sections(3) and the incubation time in the substrate was usually 12 hours because this lengthened time brought out more clearly the slight reaction in the normal thymus. For cytological studies the period of incubation was reduced to 3 and 6 hours. The technic was controlled by incubating the sections in the buffer without the substrate. The two lobes of the thymus were often treated differently for comparative studies; (1) fixed in formal-calcium(4), embedded in 25% gelatin, sectioned on the freezing microtome and colored in oil red 0; (2) fixed in acetone, embedded in paraffin and stained in periodic acid-leucofuchsin and methylene blue. Estimations of the amount of lipids were made independently by 2 or 3 persons by assigning values of + to ++++.

Observations. In studying the response of the thymus to injury the reactions of the mesenchymal and epithelial derivatives have been particularly noted. The nucleus of the former is characterized by a pattern of coarse chromatin; the latter by a large oval, vesicular nucleus, thin nuclear membrane and a fairly large bleb-like nucleolus to which is attached one or more small karyosomes. Thin cytoplasmic processes of the epithelial cells are well seen in Fig. 7 and 8.

The cortex of the normal thymus of the young BAlb/c mouse is less reactive than the medulla in all the technics used. In general there is no difference in the cortex between a subcapsular and an inner zone. There are few or no macrophages containing ingested cells, lipids or positive periodic acid-leucofuchsin granules. Only a few linear adventitial (mesenchymal reticular) cells along the capillaries contain lipids or give a positive re-

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* This investigation was supported by a research grant (C1558) from National Cancer Institute, N.I.H., U.S.P.H.S.

† The author gratefully acknowledges the assistance of Rosemarie DiMinno and Susan Brailsford Galagher.

‡ These mice were irradiated through the courtesy of the Westfield State Sanatorium, Westfield, Mass.

§ The author is indebted to Dr. Philip Ives, Dept. of Biology, Amherst College, for irradiation of the mice of this series.

action for acid phosphatase (Fig. 1). In the normal thymic medulla it is estimated that there are about 30% more lipids than in the cortex. Similarly there are more cells showing sites of acid phosphatase activity (Fig. 1). Macrophages with phagocytosed cells are scarce and small. These contain lipids and are blackened in the technic for acid phosphatase.

As soon as 2 hours after irradiation, even though the general appearance of the thymus was normal, the cells were visibly affected. In the cortex there was a limited amount of pycnosis. Phagocytosing macrophages were loaded with lipids and showed a positive reaction for acid phosphatase (Fig. 2).

At 4 hours after exposure the inner two-thirds of the cortex was more heavily laden with lipids than the subcapsular zone or medulla. In this region there were more lymphocytes and macrophages containing fat than at 2 hours and there was more evidence of acid phosphatase activity. Fig. 7 and 8 illustrate the characteristic appearance of the mesenchymal and epithelial reticular cells in the Gomori technic.

Degenerative changes were clearly evident 8 and 12 hours after irradiation. Localized cortical areas of pycnotic or fragmented nuclei gave little or no indication of acid phosphatase activity. In the inner cortical zone there were large macrophages loaded with ingested cells; these contained much lipid and gave a positive reaction for acid phosphatase (Fig. 3). In contrast to the cortex, the medulla showed little pycnosis, relatively less fat and fewer cells stained in sections treated for acid phosphatase.

At 24 hours post irradiation, the depleted and diminished cortex was heavily laden with lipids (about twice as much as at 12 hours) and stained a dark brown in the technic for for the demonstration of acid phosphatase (Fig. 4). The many loaded macrophages and epithelial cells were both colored in this reaction. Although there was a somewhat increased number of medullary cells which contained lipids and showed a positive reaction for acid phosphatase, the medulla was clearly less affected than the cortex.

Both cortex and medulla were estimated to be one-third to one-half more heavily laden with lipids 36 hours after exposure than at 24 hours and the cortex still stained deeply brown in the acid phosphatase technic. However, the removal of injured lymphocytes was indicated by the decreased amount of pycnosis and the greater number of vacuolated macrophages.

Thymuses examined 3, 5 and 7 days after irradiation showed that regenerative processes had started. At these times the amount of visible lipids and the evidence of acid phosphatase activity were diminished (Fig. 5). By 9 days the thymus was essentially normal (Fig. 6).

Discussion. Changes in lymphatic tissue after irradiation have been described mostly in relation to changes in the nuclei(5). More recently biochemical and histochemical technics have been applied to reveal other modifications in lymphatic organs after exposure to x-rays; for example, nucleic acids, Weymouth, Delfel, Doell, Steinbock and Kaplan(6); alkaline and acid phosphatases, Doyle(7,8); adenosine triphosphatase, Ashwell and Hickman(9); lipids, Smith and Holst(1).

The present study was concerned principally with the presence and distribution of histochemically demonstrable acid phosphatase in the thymus during its degenerative and regenerative phases after irradiation. The spleen and lymph node were similarly treated and examined for comparison (unpublished data). Acid phosphatase activity in the normal thymus is meager when compared with that evident in the red pulp and rim of the white pulp of the spleen or with the diffuse lymphatic tissue, medullary cords and sinuses of the mesenteric lymph node. This great difference probably results from the presence of many actively phagocytic cells in the spleen and lymph node and their scarcity in the normal thymus. As soon as 2 hours after irradiation there was an evident increase in acid phosphatase activity as the mesenchymal reticular cells became enlarged and rounded in both cortex and medulla (Fig. 2). They were, indeed, active macrophages with ingested lymphocytes. In these preparations

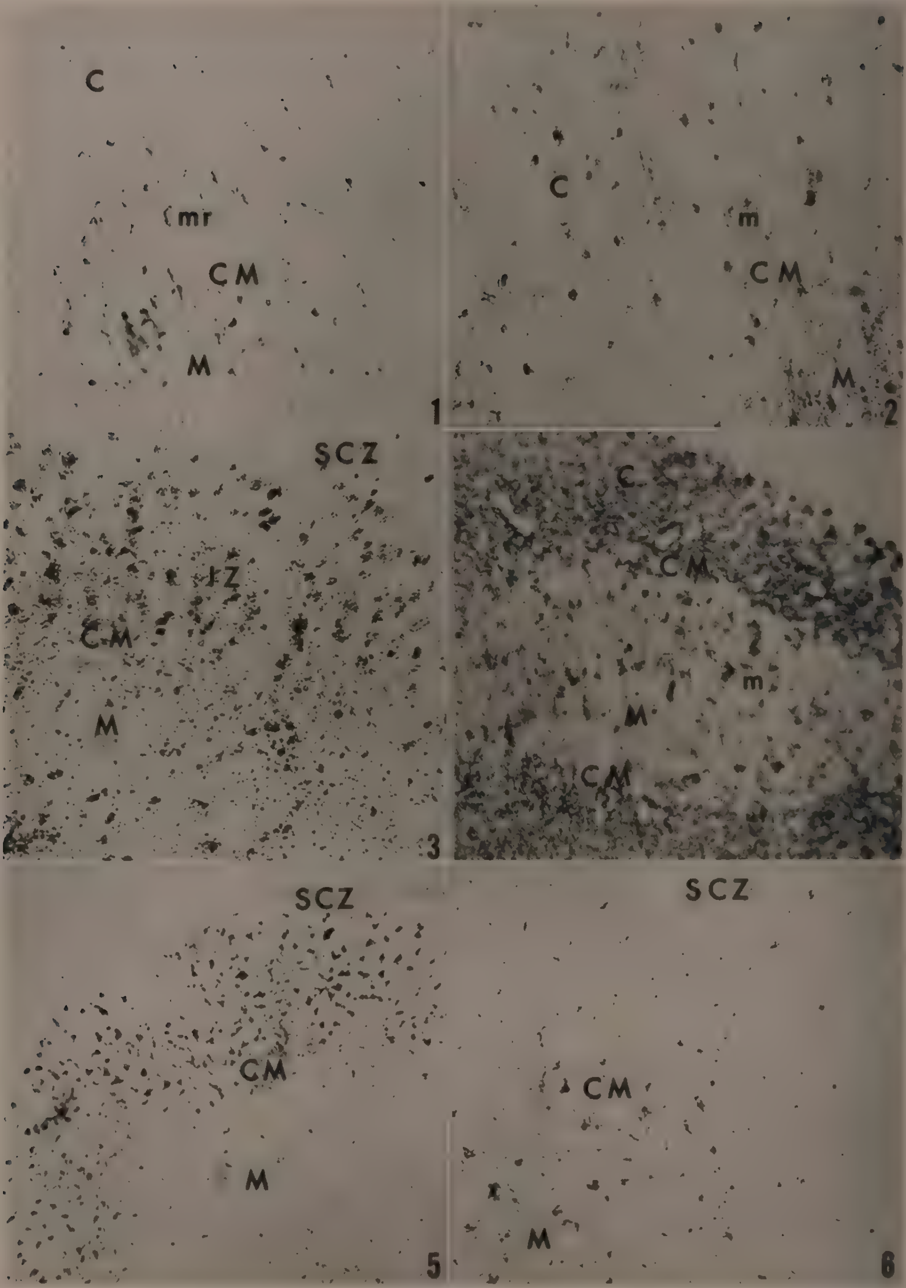
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PLATE 1

Thymus of the mouse BAlb/c; fixed in acetone; Gomori's lead sulphide method for demonstration of sites of acid phosphatase activity; 12 hr incubation. × 100.

C, cortex; CM, cortical-medullary line; er, epithelial-reticular cell; IZ, inner zone; M, medulla; m, macrophage; mr, mesenchymal reticular cell; SCZ, subcapsular zone.

FIG. 1. Control. Linear mesenchymal reticular (adventitial) cells stained in cortex; slightly more stained cells in medulla.

FIG. 2. 2 hr after total-body irradiation. Development of macrophages seen as large round cells in cortex and medulla; few linear cells seen in cortex.

FIG. 3. 12 hr after irradiation. Large loaded macrophages more crowded in cortex than in medulla.

FIG. 4. 24 hr after irradiation. Narrowed cortex loaded with stained cells. Large macrophages seen clearly at edge of subcapsular zone. Vacuolated macrophages appear as empty spaces.

FIG. 5. 5 days after irradiation. Cortex, increased in width, contains more stained cells than medulla but macrophages are smaller.

FIG. 6. 9 days after irradiation. Thymus is essentially normal in appearance.

the lymphocytes which stained were near the blackened phagocytic cells. This quick mobilization of macrophages in the thymus is indicative of the rapidity with which thymic cells react to damaging influences. Kindred (10) noted that after the use of mustard gas the cortical macrophages of the thymus were active on the first day.

The indications of greatest activity of acid phosphatase were at the height of injury 24 and 36 hours after the sublethal dose of x-rays or gamma rays, the period also of the greatest deposition of lipids. The most vulnerable part of the thymus was the cortex; its inner zone was the first to show marked signs of injury. The intense staining at these hours (Fig. 4) was due to reactive macrophages, the epithelial cells and the coloring of the associated lymphocytes. Areas where there were dense accumulations of pycnotic or fragmented nuclei were ones where macrophages were scant or lacking. As compared with the cortex the medulla was much more stable during this period. In other technics this relatively quiescent medulla results in the appearance of the so-called inverted thymus.

Summary and conclusions. 1. In the normal thymus the relatively few mesenchymal

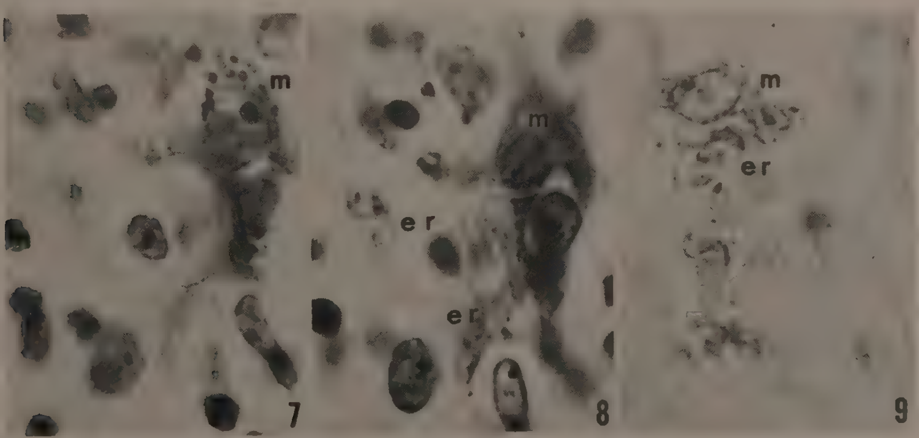

PLATE 2

Thymus of the mouse BAlb/c; fixed in acetone; Gomori's lead sulphide method for demonstration of sites of acid phosphatase activity; 6 hr incubation. × 1500.

FIG. 7 and 8. Medullary cells, 4 hr after irradiation. Same cells are photographed at 2 levels. Nuclei are stained with hematoxylin. Intimate association is seen of mesenchymal reticular cells with chromatic nuclei and stained inclusions in the cytoplasm and of epithelial cells with vesicular nuclei and weakly stained web-like cytoplasm.

FIG. 9. Medullary cells, 5 days after irradiation. A group of cells which include a macrophage with stained inclusions. Epithelial cells are barely visible. In this preparation only cells with stained cytoplasm had colored nuclei; lymphocytes and other cells with no evidence of acid phosphatase activity had unstained nuclei.

reticular cells give a positive reaction for acid phosphatase in acetone fixed sections. There are fewer reactive cells in the cortex than in the medulla. 2. As early as 2 hours after irradiation the mesenchymal reticular cells are mobilized to become active macrophages which stain deeply in the acid phosphatase technic. These become more numerous until at 24 and 36 hours after irradiation the involuted cortex is loaded with macrophages containing ingested lymphocytes or full of vacuoles. At these hours the browned cytoplasm of the epithelial cells is seen as the stroma of the depleted cortex. At 3, 5 and 7 days there is a gradual diminution in number and size of reactive cells. At 9 days the appearance of the thymus is essentially normal.

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1. Smith, C., and Holst, E. A., *Anat. Rec.*, 1953, v116, 123.
2. Lillie, R. D., *Histopathologic Technic,* 1948, Blakiston, Inc., N. Y.
3. Goetsch, J. B., Reynolds, P. M., and Bunting, H., *J. Nat. Cancer Inst.*, 1952, v13, 255.
4. Baker, J. R., *Q. J. Micr. Sc.*, 1944, v85, 1.
5. Bloom, W., *ed. Histopathology of Irradiation from External and Internal Sources,* 1948, McGraw-Hill Book Co., Inc., N. Y.
6. Weymouth, P. P., Delfel, N. E., Doell, R. J., Steinbock, H. L., and Kaplan, H. S., *J. Nat. Cancer Inst.*, 1955, v15, 981.
7. Doyle, W. L., *Am. J. Anat.*, 1950, v87, 79.
8. ———, *Symposium on Cytology,* Mich. State College Press, 1951, 1.
9. Ashwell, G., and Hickman, J., *J. Biol. Chem.*, 1953, v201, 651.
10. Kindred, J. E., *Arch. Path.*, 1947, v43, 253.

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Alterations in Calcium Metabolism in Cancer Patients Treated with 6-Diazo-5-Oxo-L-Norleucine.[†] (22741)

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6-diazo-5-oxo-L-norleucine (DON), an antibiotic isolated from culture filtrates of a Streptomyces, has been reported by Clarke *et al.* (abstract) to inhibit the growth of sarcoma 180. It is also inhibitive in mouse leukemias and a spectrum of transplantable tumors. In combination with 6-mercaptopurine (6-MP) inhibition of transplantable tumors is synergistic. Patients with cancer metastatic to bone may demonstrate hypercalcemia and hypercalcuria secondary to the osteolysis incident to tumor growth. If the tumor growth is inhibited, as, for example, by local irradiation, the calcium changes may be reversed(1) with the cessation of bone breakdown. Hypercalcemia and hypercalcuria secondary to osteolytic cancer have been corrected also by the administration of adrenal steroids(2) and other compounds. These changes have been regarded as sensitive measures of decreased osteolysis secondary to a reduced rate of growth of tumor in bone(3).

In the course of clinical trials with DON, 11 patients with hypercalcemia and/or hypercalcuria secondary to osteolytic cancer were studied in order to detect any possible tumor inhibitory effects of the compound as measured by the sensitive changes in calcium metabolism. Marked effects on calcium metabolism were seen and it is the purpose of this paper to report these changes.

Material and methods. Six patients had carcinoma of the breast, 2 metastatic carcinoma of unknown origin, 2 bronchogenic carcinoma and one adrenal cortical carcinoma. Hypercalcemia occurred 13 times in these 11 patients. Treatment with DON alone was employed in 8 instances of hypercalcemia and the combination of DON and 6-MP was used in 5 instances. One patient who was normocalcemic but hypercalcuric was treated with DON alone. All but one patient were con-

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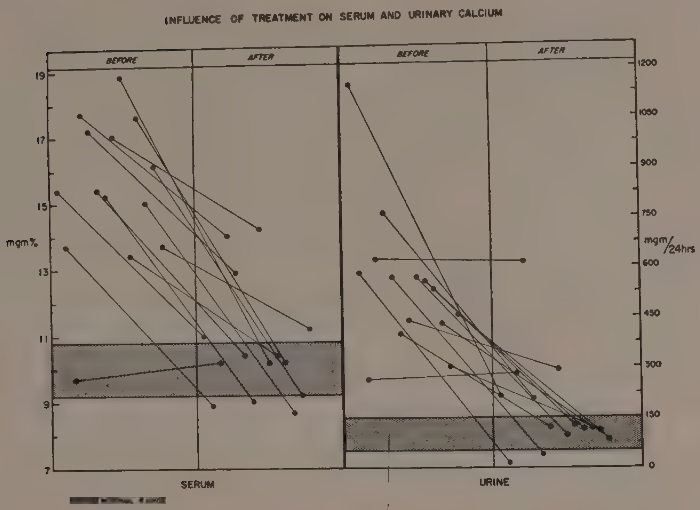

FIG. 1. Influence of treatment on serum and urinary calcium.

fined to bed and all had radiographically demonstrable metastatic cancer in their bones. DON was dissolved in saline in concentrations of 5 to 10 mg/ml. It was administered intravenously or intramuscularly in doses ranging from 0.1 mg/kg/day to 0.6 mg/kg/day. One patient received a dose of 1.2 mg/kg on one day only. Each daily dose was administered as a single injection. The duration of treatment ranged from 9 to 69 days. 6-MP was administered orally in doses of 2.5 mg/kg/day for 12-40 days. Treatment was continued in each patient until signs of toxicity developed (oral mucosal ulcerations, diarrhea, hematological depression).* Intravenous fluids had to be administered to most patients because they were too sick to retain oral feedings. Urinary calciums (method of Fiske and Logan) (4) were determined daily on 24-hour collections. Serum calciums (method of Clarke and Collip) (5) were analyzed approximately 3 times a week. The intake of calcium was restricted to 200 mg/day or less. A complete calcium balance was carried out in 2 patients. Stools and diets were ashed in a muffle furnace at approximately 600°C for 48 hours. Normal calcium ranges: Urine (on 200 mg intake) 50-150 mg/24 hours; serum 9.2-10.8 mg %. Full correction of hypercalcemia and hypercalcuria was regarded as having been achieved if the abnormal values returned to normal ranges regardless of the duration of such changes.

Results. (A) *General observations.* Fig. 1 illustrates the effect of treatment on the levels of serum and urinary calcium. The pre-treatment calcium levels represent the average of several determinations in all instances except when the serum and urinary calcium levels were rising sharply in the period before treatment. In these latter instances the highest pre-treatment value is illustrated. One patient was normocalcemic before treatment but all were hypercalcuric. All post-treatment values are the lowest ones achieved after initiation of treatment. They are not average values.

Serum. Reduction in the serum calcium occurred in each of the 13 instances of hypercalcemia although a return to normal or subnormal calcium levels occurred in only 8 instances (Fig. 1). No change from the normal range occurred in the single normocalcemic patient. The periods of treatment before these changes were seen varied from 7-26 days. The duration of the normocalcemic state was variable lasting from one day to as long as 26 days. In general, recurrences of hypercalcemia were observed when treatment

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* Unpublished data.

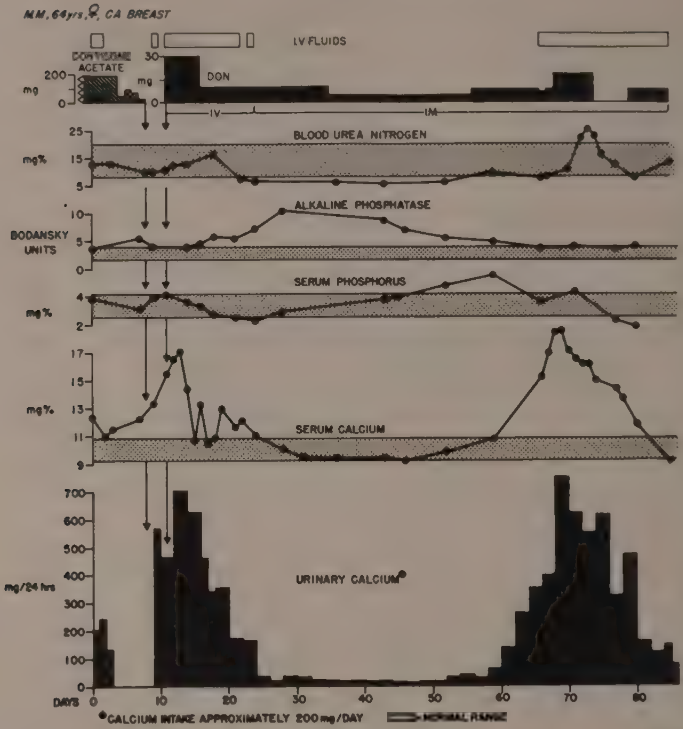

FIG. 2. Sequential changes in serum and urinary calcium in a patient with metastatic breast cancer under treatment with DON.

was stopped but the normocalcemic state persisted in some despite cessation of treatment and hypercalcemia recurred in one patient (Fig. 2) despite continuation of treatment. The decrease in the serum calcium often followed a "double-hump" pattern, that is, an initial decrease to near normal levels was followed by a rise despite continued treatment and then a fall to the normal range (Fig. 2). This pattern probably resulted from the practice of starting with high initial doses of DON (0.6 mg/kg/day) and then tapering to 0.2 mg/kg/day to avoid development of toxicity. In the 3 patients where the serum calcium decreased to lower than normal levels it rose again to the normal range although treatment was continued.

Urine. There was a decrease in the urinary calcium excretion in 12 of the 14 instances of hypercalcuria while 2 patients showed no change (Fig. 1). In 9 of these 12 instances the urinary calcium decreased to normal or lower than normal values. The pattern of response is illustrated in Fig. 2. In general the treatment periods required to produce these changes and the duration of the changes were similar to the serum calcium observations.

(B) *Treatment with DON alone.* There were three instances of full correction of both hypercalcemia and hypercalcuria. Two of these responses occurred in the same patient and are illustrated in Fig. 2. A near-complete response was observed in 2 additional patients whose serum calciums fell to 11.2 mg % and 11.0 mg % respectively with DON treatment. Definite hypercalcemia and hypercalcuria persisted in 3 patients but to a lesser degree than before treatment. There were no consistent differences in the dosages of DON received nor in the toxicity observed between those who responded and those who did not.

In general, the levels of serum and urinary calcium tended to rise promptly in both groups within 1-3 days after treatment with DON was discontinued. One patient, however, was an exception and remained normocalcemic for 22 days after cessation of DON therapy. One patient was normocalcemic but hypercalcuric (250-350 mg/24 hours) throughout the study and DON had no effect in lowering the already normal serum calcium. A total calcium balance was carried out in this patient and no effect on fecal calcium with DON treatment was noted. A total calcium balance was also carried out in another patient and no rise in fecal calcium was observed during DON therapy despite a decrease in the urinary calcium from 1148 mg/24 hours to 214 mg/24 hours over a period of 2 weeks. In one patient (Fig. 2) an attempt was made to prevent recurrence of hypercalcemia by continuing treatment with a maintenance dose of 0.1-0.2 mg/kg/day. Despite this, the hypercalcemia and hypercalcuria recurred but again were fully corrected by increasing the dose of DON.

(C) *Treatment with 6-MP plus DON*. Complete reversals of hypercalcemia and hypercalcuria were observed in 5 instances in the 4 patients in this group. The periods of treatment and total dosages of DON and 6-MP were approximately the same in each patient. In contrast to the DON group, the duration of induced normocalcemia (with one exception) ranged between 15 and 26 days and of normocalcuria between 6 and 32 days. One patient experienced only a 2 day remission in hypercalcemia but a second course of combination treatment resulted in normocalcemia lasting 19 days.

Discussion. The changes in serum and urinary calcium might be explained by a decrease in the rate of bone destruction by tumor owing to the inhibitory effects of the administered DON (and DON plus 6-MP). In view of the experimental evidences of tumor inhibition by these compounds and in view of other studies which suggest that correction of hypercalcemia is evidence of tumor inhibition in bone(1,2,3), this explanation seems most likely. The absence of roentgen evidence of bone healing is somewhat against such an interpretation and in one patient there was an actual increase in the extent of bone disease radiographically during the period between the two episodes of hypercalcemia (Fig. 2). Also disease which was present in soft tissue areas (liver, lung and subcutaneous areas) in some of the patients did not show any objective evidence of regression in conjunction with the calcium changes. All of the patients in this study succumbed to progressive disease and the transient clinical improvement noted in some was largely attributable to correction of hypercalcemia. In general, the addition of 6-MP to the treatment program seemed to exert a longer-lasting effect on the calcium changes noted. 6-MP alone has been used in 4 patients with hypercalcemia and/or hypercalcuria and no substantial effects on calcium metabolism have been observed. These observations are consistent with the demonstrated synergism between DON and 6-MP in transplantable tumors but further clinical studies are necessary in order to establish definite synergism.

Inasmuch as there was a decrease in urinary calcium excretion as the serum calcium fell rather than a calcium diuresis it is unlikely that the observed effects might have been due to enhanced renal clearance of calcium. One balance study revealed no rise in fecal calcium with DON treatment in spite of a marked decrease in urinary calcium, excluding, in this patient at least, the possibility of diversion of calcium excretion from urine to feces, as an explanation for the observed effects.

Chelation of calcium by DON was considered as a remote possibility but *in vitro* studies demonstrated no such action of the compound. Modes of action involving possible inhibition by DON of secretion of hypothetical bone-resorbing substances by the tumor remain entirely speculative at the present time.

Summary. Ten patients with hypercalcemia and hypercalcuria and 1 patient with normocalcemia and hypercalcuria were treated with 6-diazo-5-oxo-L-norleucine alone or in combination with 6-mercaptopurine.

Full correction of hypercalcemia with treatment occurred in 8 of 13 instances in these patients. Correction of hypercalcuria was observed in 9 of 14 instances. Various possible mechanisms underlying these observed effects are considered.

1. Albright, F., and Reifenstein, E. C., Jr., *The Parathyroid Glands and Metabolic Bone Disease*, Williams and Wilkins, Baltimore, 1948.

2. Pearson, O. H., Li, M. C., Maclean, J. P., Lipsett, M. B., and West, C. D., *Annals N. Y. Acad. Sci.*, 1955, v61, 393.

3. Pearson, O. H., West, C. D., Hollander, M. D., and Treves, N. E., *J.A.M.A.*, 1954, v154, 234.

4. Fiske, C. H., and Logan, M. A., *J. Biol. Chem.*, 1931, v93, 211.

5. Clarke, E. P., and Collip, J. B., *ibid.*, 1925, v63, 461.

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Effect of Sodium Lack on Local Response of the Single Crustacean Motor Axon. (22742)

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Sometime ago it was suggested, as the basic assumption for a theory on excitation of nerve (1), that a local or initial depolarization occurred in the immediate area of nerve to which the stimulus was applied. When this local voltage attained a certain optimum value it released or "triggered" the all or none response. Shortly thereafter this hypothetical local potential was measured directly(2) and examined in some detail(3) in crustacean nerve fibers. However, the actual source of this small potential remained obscure. Recently the all or none response has been studied quantitatively by these same investigators and others(4,5), and it has been established that the action potential is the result of an initial rapid sodium ion current inward through the fiber membrane followed by a slower outward potassium ion flow. The question now remains what is the cause of the small local disturbance always observed in the cathodal region with subthreshold stimulation? Is this also due to ionic currents or is it perhaps the result of the sudden appearance of a hormonal substance? It has been suggested, for example, that the release of ACH might produce the initial or local depolarization(6) as it does an end plate potential at the neuromuscular junction.

The present work was undertaken to examine the local response of different single crustacean nerve fibers which possessed quite different excitability properties(7). A brief report of this investigation has already been given in abstract. Recently, certain results were obtained which, because of their significance, are described briefly in this paper.

Methods. The lobster single nerve fiber was used throughout. The preparation, mounting and recording technics have been described (8). The local or initial response was clearly observed with subthreshold stimulation when the fiber was entirely raised in oil, the V method, and stimulation and recording occurred in the identical cathodal area. Cole's physiological solution(9) was used with variations for the study of the effect of different ion concentrations.

Preliminary. One day by accident in mixing the physiological solution the buffer was omitted and although the pH was very low, found later to be only 5.8, the nerve fibers functioned well for several hours. Others, too, have noticed the lack of effect of low pH on these fibers (Adelman, personal communication). The importance of this finding however, was that it meant an entirely sodium-free solution could be applied to a nerve fiber, for some slight amount of sodium has always necessarily been included in the form of buf-

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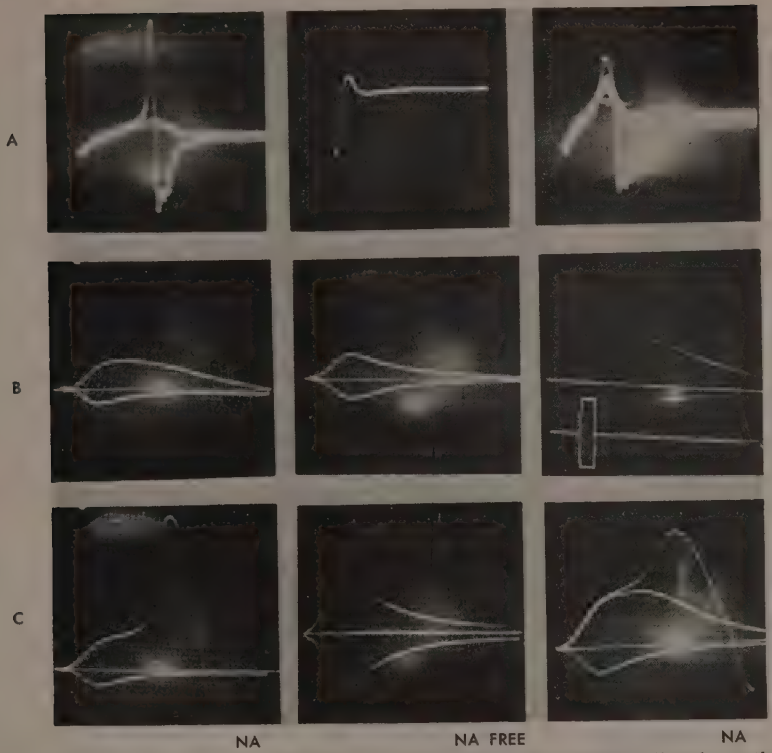

FIG. 1. Electrical response of a single crustacean opener fiber recorded directly at the stimulating cathode. Row A—Response with long duration, 100 ms, stimulation in normal physiological solution, left, sodium free (choline chloride substituted), middle, and normal solution, right. Row B—Recordings with short duration, 0.2 ms, stimuli of negative (upward) or positive (downward) polarity. Note asymmetry, negative deflection much larger in left and right pictures. In sodium free solution, middle picture, deflection is symmetrical. Row C—Same as B with stronger, threshold, stimulus. See text.

fers in so called "sodium free" media.

Results. When a single nerve fiber was dipped into a sodium free solution, containing dextrose or Choline Chloride to maintain the proper tonicity, all nerve electrical activity was extinguished instantly. Not only was the all or none spike immediately abolished, a not unexpected observation, but the subthreshold local response also disappeared at once. The effect was dramatically sudden and complete. With sodium ions added even in small concentrations the local response reappeared as abruptly as it disappeared in the sodium free medium. The recovery of the all or none potential was also extremely rapid but depended somewhat on the amount of sodium placed in the solution. When only a small concentration of sodium was added to the medium the spike appeared, not unlike the spurious response of a fatigued fiber.

A typical experiment is shown in Fig. 1. In the top row, A, the local and spike potentials elicited by "D.C." stimulation of 100 ms. duration are shown in normal, sodium free, and normal solutions, left to right respectively. The sweep speed for these recordings was slow. In the middle row, B, are illustrated the responses to just subthreshold, 0.2 ms. duration shocks. The sweep speed was fast. The bottom row, C, illustrates the recordings with threshold stimulation of 0.2 ms. duration in normal, sodium free and normal solutions, left to right respectively. The short duration shocks applied were both negative, upward deflection, and positive, downward deflection, polarity as shown in the right picture, row B. A just subthreshold negative shock should produce a much larger deflection than a positive shock, due to the local response of the fiber itself in the neighborhood of a cathode(3).

At the start of the experiment with normal solution and D.C. stimulation there was a low voltage long duration subthreshold response and a large diphasic spike with threshold voltage. In the sodium free medium with strong stimulation there was a slight artifact but no real electrical activity. With sodium in the medium again, right hand picture, with varying strengths of stimulation, multiple sweeps, a large amplitude fairly short duration local response is the predominant deflection observed which when large enough produced an all or none response somewhat smaller and longer enduring than the original. Short duration subthreshold pulses normally elicited asymmetrical deflections, that is, the upward or negative deflection was far greater than the downward or positive deflection as expected (Row B, left picture). The negative deflection is augmented by the activity of the fiber, the local response, the positive deflection is not. The local response, catelectrotonus, of the fiber is responsible for the asymmetry of the deflections. These recordings are the same as described years ago(3). This asymmetry was also observed with threshold stimulation. However, with sodium free solution the responses due to positive and negative pulses, weak or strong, became perfectly symmetrical. The local response was gone, and only the shock artifacts of equal magnitude remained. The fiber was electrically totally inactive, or, to all intents and purposes "dead". With the return of sodium to the medium the action potential, slightly smaller and broader, appeared immediately and at the same time a subthreshold response of even greater asymmetry than at the start was recorded. This effect has been seen in all fibers used.

Conclusion. Sodium is necessary for the production of the local response. It has yet to be proven just what the role of the sodium ion is in the development of this local potential. The above findings do not eliminate the possibility of the release of a hormone such as ACH as the "trigger" mechanism, for it has been shown(10) that end plate potentials are all but abolished in the presence of ACH without sodium. However, it seems very likely that the sodium ion is the key to the setting off and development of the action potential. This has been known to be so for whole frog nerve preparations(11). Whether the ion actually moves inward to cause the local potential as it does during the rising phase of the spike remains to be determined. Its presence may simply be required for the initiation of some other reaction. The fact that the results are the same whether dextrose or Choline Chloride is substituted indicates that it is the sodium ion itself that is the essential particle.

Summary. The local or initial response of crustacean single motor axons has been recorded at the stimulating cathode. The deflections caused by a short duration shock of positive and negative polarity are asymmetrical. Normally the deflection caused by the negative pulse is considerably larger. In the absence of sodium, with either dextrose or Choline Chloride substituted, the local potential disappears. The deflections caused by positive and negative pulses become symmetrical. The effect is instantaneous and reversible. It is concluded that the sodium ion is essential for the production of the local or initial response.

1. Hill, A. V., *Proc. Roy. Soc.*, 1936, v119, 305.
2. Katz, B., *ibid.*, 1937, v124, 244.
3. Hodgkin, A. L., *ibid.*, 1938, v126, 87.
4. Hodgkin, A. L., and Katz, B., *J. Physiol.*, 1949, v108, 37.
5. Hodgkin, A. L., and Huxley, A. F., *ibid.*, 1952, v117, 500.
6. Nachmanson, D., and Wilson, I. R., *Electrochemistry in Biology and Medicine*, Wiley and Sons, 1955.
7. Wright, E. B., and Coleman, P. D., *J. Cell. Comp. Physiol.*, 1954, v43, 133.
8. Wright, E. B., Coleman, P. D., and Adelman, W. J., *ibid.*, 1955, v45, 273.
9. Cole, W. H., *J. Gen. Physiol.*, 1941, v25, 1.
10. Nastuk, W., *Am. J. Med.*, 1955, v19, 667.
11. de No, L., *Studies from Rockefeller Inst. for Med. Research*, 1947, v132, part I.

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Propagation *in vitro* of Polioviruses. VIII. Effect of pH on Virus Yield and Cell Metabolism.* (22743)

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Susceptible animal cell cultures infected by any of a variety of cytolytic viruses undergo progressive cellular degeneration(1). The pH of the culture medium is decreased concomitantly by cell metabolic activity. The purpose of this study was to measure quantitatively the effects upon virus yield and upon host cell metabolism of purposeful alterations in pH of the medium. Strain HeLa cells and poliovirus, Type 1, strain Mahoney, were employed to learn the effect of increments in pH from 6.3 to 8.0.

Materials and methods. Host cells: Strain HeLa cells were employed, cultivated, and maintained as described(2). The growth medium was composed of 40% human adult serum and 60% Hanks' balanced salt solution (HuS-40, BSS-60). *Assay for virus*: Virus titrations were conducted with 5 replicate HeLa cell cultures at each successive geometric dilution. Titers computed as most probable numbers of tissue culture infectious units (TCIU) from the frequency of positive tubes agreed within the range ± 0.3 log. For virus titrations the human serum growth medium was removed from cell cultures by 3 rinsings with BSS and replaced with 10% chicken serum, 90% modified maintenance solution (MMS) medium(3). *Virus.* Poliovirus, Type 1, strain Mahoney, was employed as supernatant fluid from passage 65 in cultures of HeLa cells, stored at —70°C. A small amount of virus (usually 15 to 30 TCIU) was employed in each experiment so that several cycles of virus synthesis could be followed and thus small effects would be magnified. *Manometric method.* The procedure for the cultivation of HeLa cells in Warburg flasks and the measurement of respiration has been described(4). *Glucose determination.* The Nelson-Somogyi photometric method of estimating glucose was employed(5). Readings were made in a Beckman DU spectrophotometer at 650 mμ. *Buffers.* For pH studies the MMS medium was fortified with additional buffers. Sodium hydrogen phosphite(4), 0.01 M, tris (hydroxymethyl) aminomethane (THAM), 0.01 M and sodium hydrogen phosphate, 0.01 M were employed. When buffers were employed an equivalent molar concentration of sodium chloride was omitted from the medium so as not to increase tonicity. The required pH was obtained by the addition of 0.15 M hydrochloric acid or sodium hydroxide.

Results. Effect of pH on poliovirus yield in stationary HeLa cultures. The first experiment was designed to test the effect of pH in range from 6.3 to 7.3 upon the replication of poliovirus in stationary cultures of strain HeLa cells. Supplementary buffers were found necessary to maintain the pH. Several buffers were tested for toxicity for strain HeLa cells. Table I shows that phosphate, 0.02 M, phosphite, 0.02 M and THAM, 0.01 M, did not significantly alter the respiration of HeLa cells over an observation period of 78 hours. Medium, ChS-10, MMS-90 containing 0.01 M each of phosphite, phosphate and THAM was prepared. Aliquots of the medium were adjusted to the required pH by the addition of 0.15 M HCl or NaOH, and were made up to equal volume

TABLE I. Effect of Various Buffers on Respiration of Strain HeLa in ChS-10, MMS-90.*

		Oxygen uptake, μl		
		Hr of incubation		
Buffer	Molarity	24	53	78
Control		212	475	751
Phosphate	.02	232	540	783
Phosphite	.02	235	550	800
THAM	.01	225	535	810

* 840,000 cells/flask.

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TABLE II. Effect of Variable pH upon Poliovirus Yield in Strain HeLa Cell Cultures.

pH			log TCIU/ml	
Initial	24 hr	37 hr	24 hr	37 hr
6.3	6.3	6.3	2.0	2.0*
6.5	6.5	6.5	2.5	3.5†
6.7	6.7	6.7	3.5	5.1‡
6.9	6.9	6.9	3.7	6.0
7.1	7.05	7.0	4.0	6.1
7.3	7.25	7.2	4.1	6.0

* Control bottles without virus demonstrated toxicity; over 50% of cells off glass, remaining cells rounded and granular.

† Control bottles without virus demonstrated toxicity; approximately 25% of cells off glass, remaining cells rounded and granular.

‡ Control bottles without virus demonstrated toxicity; few cells off glass, some rounding of cells.

with 0.15 M NaCl. Approximately 3.0 mg/ml of glucose were employed in the medium. Duplicate planar bottle cultures of strain HeLa were prepared at each pH increment and infected with approximately 25 TCIU of poliovirus. All cultures were incubated at 36°C. To better maintain the pH, 20% of the medium was replaced every 6 hours. The experiment was terminated at the first evidence of cytopathogenicity so that variations in the number of HeLa cells would not limit virus yields. Results of titrations of supernatant fluids harvested at 24 and 37 hours are shown in Table II. The virus yield from cells incubated at pH 6.7 or lower was significantly retarded or reduced. The possibility existed that decreased yield of virus observed at the lower pH levels might be due to a greater instability of the virus at these pH levels. Aliquots of media at pH 6.5, 6.8, 7.0 and 7.5 were prepared with $10^{3.8}$ TCIU/ml of virus and allowed to incubate at 36°C for a period of 42 hours. Titrations to determine the stability of poliovirus at these pH levels were performed. Titers all fell within the range $10^{3.5 \pm 0.3}$ TCIU/ml of virus. Maintenance of the original titer indicated that the virus was stable at the pH increments employed. *Effect of pH on HeLa cell metabolism.* To determine what effect alteration of pH had upon the metabolism of HeLa cells, quadruplicate Warburg flasks were prepared at each of 4 pH levels and 2 flasks of each series were infected with approximately 25 TCIU of virus. Medium, MMS-90, ChS-10, fortified with 0.01 M each of phosphate, phosphite and THAM, was employed. The results of three separate experiments are summarized in Table III. With alterations in pH from approximately 7.5 there resulted a decrease in oxygen uptake and a decrease in glucose utilization. The results at an initial pH of 6.9, 7.6 and 8.0 are shown in Fig. 1.

Discussion. Results of these experiments showed that the yield of poliovirus, Type 1, Mahoney strain, in HeLa cells was dependent upon the pH of the medium in which the host cells were maintained. The decreased yield of virus at decreased or increased pH levels was related to a decreased metabolic state of the cell cultures; such cultures failed to be maintained as shown by the decreasing rate of oxygen uptake. Fogh(6) investigated the effects of pH variation on absorption of poliovirus by monkey kidney cells; he concluded that possible differences in virus absorption at pH levels between 6.0 and 8.0 did not materially affect the latent period or virus yield. This evidence and evidence for the stability of poliovirus further supports the conclusion that reduced virus yield at low or high pH levels results from a decreased metabolic rate of the host cell. The present experiments also emphasize the importance of pH as a factor affecting metabolic rate of host cells and thus of virus yield. It is apparent that

TABLE III. Effect of Alterations in pH upon Poliovirus Yield and upon Metabolic Activity of Strain HeLa Cell Cultures.

pH		Glucose utilized,* μg/ml/10 hr/100,000 cells	Oxygen uptake,* μl/10 hr /100,000 cells†	Virus yield, log TCIU /ml
Initial	Final			
6.6	6.6	0	4.3	3.8
6.8	6.6	8	5.6	4.9
7.0	6.8	15	6.6	5.7
7.2	6.9	18	9.0	6.4
7.1	7.1	21	8.0	6.6
7.4	7.3	24	8.7	6.4
7.6	7.4	21	9.5	6.4
7.7	7.5	21	10.0	6.6
7.7	7.6	28	10.0	6.2
7.8	7.6	28	9.8	6.4
7.9	7.7	33	9.5	6.0
8.0	7.8	13	7.9	5.1

* Avg value obtained over period of observation.

† Initial live cell population.

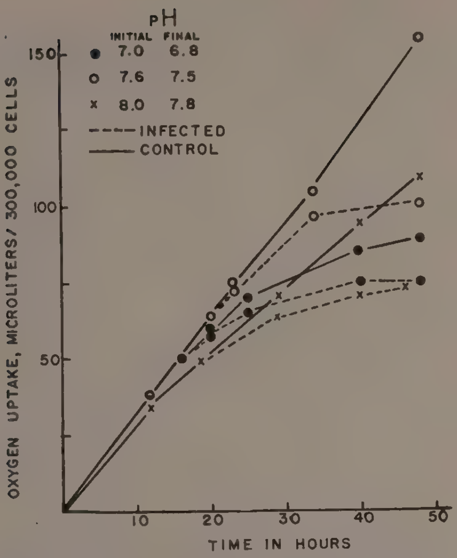

FIG. 1. Oxygen uptake of infected and control HeLa cell cultures at initial pH of 7.0, 7.6 and 8.0.

virus titrations should be carried out in adequately buffered media and that reduction of medium pH below 7.0 during incubation should be corrected. It is also apparent that in screening potential chemotherapeutic or chemoprophylactic agents the effect of pH should be considered. A test agent could act upon cells to increase glycolysis. The resultant increased acid production would thus lower virus yield to simulate specific interference with virus production.

Summary. The pH of tissue culture medium was altered purposefully to measure quantitatively the effects upon virus yield and host cell metabolism. By use of strain HeLa cells and poliovirus Type 1 (strain Mahoney) for growth in media of pH from 6.3 to 8.0 it was established that virus yield was affected by the pH of the culture medium. Decreased virus yield at decreased or increased pH levels was related to a decrease in metabolic rate of host cell cultures.

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1. Enders, J. F., *Ann. Rev. Microbiol.*, 1954, v8, 473.

2. Syverton, J. T., and Scherer, W. F., *Ann. N. Y. Acad. Sci.*, 1954, v58, 1056.

3. Gifford, G. E., Robertson, H. E., and Syverton, J. T., *J. Cell and Comp. Phys.*, 1956.

4. Gifford, G. E., Robertson, H. E., and Syverton, J. T., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 515.

5. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

6. Fogh, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 494.

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Chronic Catheterization of the Renal Artery: Technic for Studying Direct Effects of Substances on Kidney Function.* (22744)

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During the course of studies on the pathogenesis of sodium retention in experimental congestive heart failure(1) it was shown that sodium retention occurred in dogs with normal basal glomerular filtration rates, suggesting the presence of increased tubular reabsorption of sodium. In order to obtain more direct information concerning the increased tubular reabsorption of sodium, a method has been developed for chronic catheterization of one renal artery, with collection of urine from each kidney individually. Such a technic affords the opportunity of studying the direct effect of salt solutions, hormones and drugs injected directly into the renal artery of the unanesthetized dog, using the opposite kidney as a control. Furthermore such experiments are relatively uncomplicated by extrarenal factors, and allow the separation of renal from

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extrarenal effects in the unanesthetized dog. The presence of the catheter in the renal artery has not altered the glomerular filtration rate of that kidney, the renal plasma flow, the daily urine volumes, daily sodium excretions, or the response of the kidney to intravenous loads of sodium chloride.

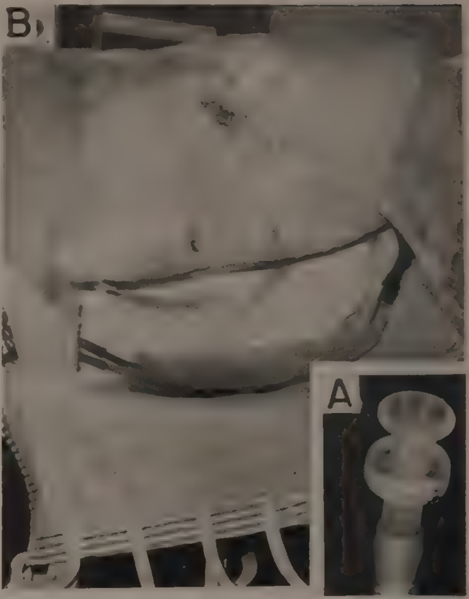

FIG. 1. A. Lucite funnel for urine collection. B. Illustration showing plastic bags attached to lucite funnels, and renal artery catheter secured to skin suture on flank of dog.

Methods. Technic of urine collection from each kidney individually is a modification of the method described by Desautel.[§] The dog, under sodium pentobarbital anesthesia, is placed in a supine position. Through a midline suprapubic incision the urinary bladder is exposed, and drained by needle puncture. The neck of the bladder is mobilized and the urethra cut between two heavy ligatures. The bladder is then hemisected in the sagittal plane, starting on the anterior surface, continuing up over the fundus and posteriorly down between the ureteric orifices. The hemisected bladder is then reconstructed into two separate bladder pouches. Each half of the bladder is sutured around a lucite funnel (Fig. 1A). Starting at the urethral end of the bladder the serosal surfaces of each half of the bladder are brought together by mattress sutures, inverting the bladder mucosa; a similar inverting suture is started at the fundal end of the bladder and continued down toward the midportion of the cut surface. The cup of the funnel is inserted into the bladder pouch thus formed and the sutures are continued up to the threaded stem of the funnel. A purse-string suture in the serosa is then drawn tightly around the funnel stem. The stem of each funnel is brought through a stab wound in the abdominal wall 2 to 3 cm lateral to the midline. Omentum is then sutured between the two pouches to reduce the likelihood of fistula formation, and the abdominal incision is closed. A lucite-nut with smoothed edges is screwed on to the stem of the funnel and tightened down to the abdominal wall to hold the bladder snugly against the anterior abdominal wall. A length of rubber tubing is attached to each funnel and is in turn connected to the plastic bags for urine collection (Fig. 1B). Rubber tubing is also attached to the distal end of the urine bags and closed with Hoffman clamps, which allow fixation to the inside of the canvas jacket which is worn by the dog. The sterilized urine bags are changed daily. After a 7 to 10-day recovery period creatinine, para-amino-hippurate and sodium clearances are measured to assess any possible changes in these functions resulting from the subsequent catheterization of the renal artery. *Technic for implantation of catheter in renal artery.* The dog, anesthetized with sodium pentobarbital, is placed in the right lateral decubitus position. A left flank incision is made below the costal margin and the left kidney exposed. Blunt dissection in the retro-peritoneal space allows mobilization of the aorta for 2 inches above and below the left renal artery. The operation is abandoned if more than a single renal artery supplies the left kidney. Light rubber tubing is passed around the aorta, above and below the root of the renal artery, for counter traction and control of blood loss during subsequent manipulation. A 10-inch length of smooth, polyvinyl catheter is used. This catheter has an inside diameter of .015 inch and an outside diameter of .028 inch with a total volume of approximately .05 ml. A stainless steel wire is threaded through the catheter to the tip to facilitate handling of the catheter. A purse-string suture is placed on the postero-lateral aspect of the aorta near the origin of the renal artery. The wall of the aorta is then perforated through this purse-string suture with a sharp needle. While countertraction is applied to the rubber tubes around the aorta, a blunt thin-walled curved needle, with a removal plug in the hub, is passed into the lumen of the aorta and across into the orifice of the renal artery and advanced approximately 1 cm. The plug is then withdrawn and the catheter passed through the needle and gently advanced beyond the end of the needle. The needle is cautiously withdrawn while the catheter is held firmly in place. The stainless steel wire is then removed. The purse-string suture around the catheter is then tied for hemostasis. The catheter is flushed with heparin and the external tip clamped. Branching of the renal artery usually occurs within the first inch of its origin from the aorta. To insure the perfusion of the entire kidney with injected substances, the catheter must be withdrawn until its tip lies proximal to the first bifurcation. Palpation of the catheter in the renal artery is not always reliable. Indigo carmine, injected through the catheter, produces a short-lived dark blue discoloration of the portion of the kidney through which the dye passes and sharply delineates the tissues not perfused with the dye. The catheter is then withdrawn 2 or 3 mm after each injection of indigo carmine until a single injection perfuses the entire kidney, indicating that the catheter tip now lies in the main stem of the renal artery and proximal to any bifurcation. The discoloration of the entire kidney also indicates that adequate mixing occurs in the renal artery. The catheter is then secured to the aortic wall. A small loop is left in the retroperitoneal space with further fixation to muscle and subcutaneous tissue. A second loop is left in the subcutaneous tissue and the external tip brought through the skin. The catheter is flushed with concentrated heparin (10,000 units per ml) and closed with a stainless steel obturator inserted into the tip. A fine tie is placed on the catheter over the metal obturator and this is secured to a loop sutured in the adjacent skin. A small protective covering is applied over the end of the catheter before the jacket is drawn on to the animal. The catheter is flushed each day with heparin.

[§] To be published.

TABLE I. Representative Daily Urine Volume (ml) of the 2 Kidneys before and after Chronic Catheterization.

Preoperative		Postoperative	
Left	Right	Left	Right
390	380	360	305
380	365	175	140
142	145	255	225
290	287	370	320

Results. Successful bladder division and catheter implantation has been performed in 5 normal dogs and 2 dogs with right-sided congestive failure. Such animals have now been followed for periods as long as 5 months without functional or pathological evidence of renal damage.

The ratios of the daily urine volumes of the 2 kidneys have not been altered by the insertion of the catheter into the left renal artery (Table I). Furthermore, the presence of the catheter has not modified the creatinine or par-aminohippurate clearances. In a representative dog (Table II) control creatinine clearances of the left kidney varied from 30 to 38 ml/min; after catheterization values ranged from 29 to 31 ml/min differences which are of the same order of magnitude observed in the right or control kidney. The para-aminohippurate clearances of the left kidney varied from 82 to 89 ml/min before,

TABLE II. Representative Creatinine and Para-aminohippurate Clearances (ml/min.) before and after Chronic Catheterization.

C_{CR}				C_{PAH}			
Pre-operative		Post-operative		Pre-operative		Post-operative	
Left	Right	Left	Right	Left	Right	Left	Right
36	32	29	28	82	79	83	85
38	37	30	31	89	87	90	94
31	28	31	33	85	80	94	95
30	28	31	32	85	81		

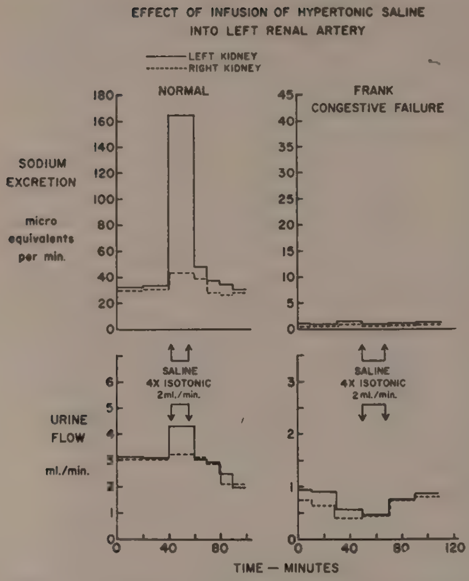

FIG. 2. Effect of infusion of hypertonic saline into left renal artery of normal dog, and dog with congestive heart failure.

and 83 to 94 after, catheterization. Repeated clearance measurements over a period of 5 months have shown no significant changes. The animals were sacrificed at varying intervals after catheterization; neither the control nor catheterized kidneys showed any histological abnormalities. The bladder mucosa was hyperemic and hypertrophied.

With the successful development of the technic for chronic catheterization of the renal artery preliminary studies have been performed on renal tubular reabsorption of sodium in normal dogs and in dogs with chronic congestive heart failure. For example, as indicated in Fig. 2, the infusion of hypertonic saline (580 meq/L) into one renal artery of a normal dog at the rate of 2 ml/min produces a 5 to 10 fold increase in sodium excretion on the injected side with a smaller increment in water excretion. Little or no change is observed in the control kidney. A similar infusion into the renal artery of a dog in congestive failure, with marked sodium retention but a normal basal glomerular filtration rate, does not produce any increase in sodium or water excretion—further evidence for increased tubular reabsorption of sodium and water in congestive heart failure.

Summary. A method has been developed for chronic catheterization of one renal artery, with collection of urine from each kidney separately. The presence of the catheter has not altered renal function over a period of months. Preliminary studies with infusion of hypertonic saline into the renal artery of the dog in congestive heart failure indicate a marked increase in tubular reabsorption of sodium.

1. Barger, A. C., Yates, F. E., and Rudolph, A. M., *Am. J. Physiol.*, 1955, v183, 595.

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Adenosine and Ash as Unidentified Chick Growth Factors in Fish Meal.* (22745)

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Growth stimulation of many microorganisms by adenine and other purines has long been recognized(1). It has also frequently been postulated that in animal nutrition it may be possible that the rate of purine synthesis is inadequate for optimal growth, and at various times adenine and its derivatives have been stated to possess vitamin-like properties. For example, vit. B_4 has been postulated to be adenine and vit. L_2 has been stated to be adenylthio-methyl pentose. Huff and Bosshardt(2) have reported that the growth retardation brought about by the inclusion of succinylsulfathiazole in the diet of mice is

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largely prevented by the addition of adenine and by other purines and purine ribosides. Very recently(3) it has been reported that adenosine stimulates the growth of chicks fed a purified diet. A number of reports indicate that the ash of various natural products will, under certain conditions, stimulate the growth of chicks on purified diets(4-6) and corn-soya type rations(7). In contrast, negative results from alfalfa ash(8) and the ash of distillers' dried solubles(9) have been reported with purified diets where the salt mixture used in the present work was employed.

Fractionation work on the unidentified growth factor(s) in fish meal has been under our investigation for some time. We are presenting evidence at this time that the organic factor in fish meal is not adenosine and that supplemental adenosine does not improve our basal diet which gives excellent growth in contrast to the growth reported in the study referred to above. The fish meal† employed has consistently given a marked growth response when added to a purified basal diet containing an isolated soya protein.

Methods. Male chicks from a mating of New Hampshire males x Columbian females were used throughout. The parent stock had received a diet containing liberal amounts of fish meal, whey, and alfalfa meal. The chicks were housed in electrically heated batteries with raised wire floors. They were offered food and water *ad libitum* from one day of age. In each experiment three or four replicates of 10 chicks were used for each treatment. The experimental ration is given in Table I. The fish meal ash was prepared by ashing in a muffle furnace at a dull red heat and the ash was fed at a level equivalent to the ash in 10% fish meal. This amounted to 2% ash added to the ration. All supplements were added at the expense of cerelose. The general experimental design of the 3 experiments reported here can be seen from Table II. In Exp. 2 the Glista salt mixture was increased from the usual 5.34% to 7.34% in group 4, and in group 5 it was decreased to 3.34% so that the 2% fish meal ash replaced 2% of the usual mineral mixture. In Exp. 3 adenosine was fed both as the only supplement to the diet (group 4) and also on top of added fish meal (group 5) and on top of added fish meal ash (group 6).

TABLE I. Composition of Basal Diet.

	%
Cerelose	57.11
Drackett protein C-1	35.30
Salt mixture*	5.34
Corn oil (refined)	1.00
DL-methionine	.75
Glycine	.30
Choline Cl	.20
Vitamins†	+
Total	100

* See reference 8.

† Vitamins (mg/kg diet):

Thiamine HCl	100	Inositol	100
Niacin	100	P-aminobenzoic acid	2
Riboflavin	16	Ascorbic acid	250
Ca-pantothenate	20	Menadione	5
B_{12}	.02	α-toc. acetate	20
Pyridoxine HCl	6	Vit. A acetate	10,000 I.U.
Biotin	.6	D_3	600 I.C.U.
Folic acid	4		

TABLE II. Growth Response of Chicks to Various Supplements.

Group ration	Avg wt (g) 28 days	% increase over basal
Exp. 1		
1. Basal	280*	—
2. " + 10% fish meal	355	26.8
3. " + 2% fish meal ash ≎ 10% fish meal	307	9.6
Exp. 2		
1. Basal	381†	—
2. " + 10% fish meal	438	15.0
3. " + 2% " " ash	389	2.1
4. " + 2% Glista salts	390	2.4
5. " − 2% Glista salts + 2% fish meal ash	395	3.7
Exp. 3		
1. Basal	365*	—
2. " + 10% fish meal	425	16.4
3. " + 2% fish meal ash ≎ 10% fish meal	379	3.8
4. " + adenosine 150 mg/kg	346	−5.2
5. " " + fish meal (2 + 4)	414	13.4
6. " " + ash (3 + 4)	358	−1.9

* Avg of triplicate groups of 10 chicks each.
† " " quadruplicate " " " " " .

Results. From the results presented in

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† A defatted fish meal prepared by VioBin Corp, Monticello, Ill., and generously supplied by Mr. Ezra Levin.

Table II it appears evident that adenosine gave no growth response when added to the soy protein type synthetic ration used in this study, or when added to this diet on top of fish meal or fish meal ash supplements. The picture with regard to ash is not quite so clear but certainly, considering all experiments, little response was obtained compared to the response obtained by the addition of 10% of this particular sample of fish meal. Other experiments not reported here have given the same order of response with respect to the intact fish meal.

Summary. It was found that adenosine at 150 mg/kg of ration failed to stimulate chick growth when supplementing a 30% protein, soy protein type purified diet. Supplementation of such rations with 2% fish meal ash gave only small and variable responses, while supplementation with 10% fish meal uniformly gave marked growth response.

1. Snell, E. E., and Mitchell, H. H., *Proc. Nat Acad. Sci.*, 1941, v27, 1.

2. Huff, J. W., and Bosshardt, D. K., *J. Am. Chem. Soc.*, 1952, v74, 4472.

3. Barnett, B. D., Lapidus, M., Bird, H. R., and Strong, F. M., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 372.

4. Morrison, A. B., Scott, M. L., and Norris, L. C., *Poultry Sci.*, 1955, v34, 738.

5. Dannenburg, W. N., Reid, B. L., Rozacky, E. E., and Couch, J. R., *ibid.*, 1955, v34, 1023.

6. Tamimie, H. S., *ibid.*, 1955, v34, 1224.

7. Menge, H., Lillie, R. J., Sizemore, J. R., and Denton, C. A., *ibid.*, 1956, v35, 244.

8. Fisher, H., Scott, H. M., and Hansen, R. G., *J. Nutrition,* 1954, v52, 13.

9. Scott, H. M., Morrison, W. D., and Griminger, P., *Poultry Sci.*, 1955, v34, 1446.

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Effect of Replacement Therapy on Dental Caries Experience of Radiothyroidectomized Rats.* (22746)

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Previous work from this laboratory(1-3) has indicated that the thyroid gland in rats is associated with the dental caries experience. By feeding desiccated thyroid it has been possible to repeatedly demonstrate a significant reduction in dental caries while the administration of I^{131} or thiouracil results in a significant increase in dental caries. From these data it appears quite convincing that the thyroid gland is intimately associated with the dental caries experience in rats. It has become a difficult problem to determine accurately the mechanism by which these effects take place. Various facts are known, however, which suggest that the salivary glands are important intermediates in this mechanism. For example, when salivariadenectomized rats are used, there is no appreciable anticariogenic effect observed by feeding desiccated thyroid. Also, it has been possible to demonstrate a direct relationship between thyroid function, the histological structure of the submaxillary gland and the incidence of dental caries. A further correlation has been shown not only between the microscopic structure of the submaxillary glands, their proteolytic enzyme content and dental caries incidence in hypophysectomized rats but also of proteolytic activity and arginase activity in the submaxillary glands with thyroid function(4-6).

To extend these studies on the relationship of the rat thyroid gland and dental caries, it was considered important to determine if it were possible to return the increased incidence of dental caries occurring in radiothyroid-

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TABLE I. Comparison of Incidence of Dental Caries in Radiothyroidectomized Rats Receiving Thyroxine and Testosterone.

Group	Sex	No. of animals	Final wt (g)	No. of lesions		Probability*	
Control	♂	21	370	8.9	> 8.8	—	.001
	♀	18	225	8.7			
I^{131}	♂	18	185	12.7	>13.5	.001	—
	♀	19	131	14.3			
Control + thyroxine	♂	16	326	6.8	> 6.1	.05	.001
	♀	17	242	5.4			
Control + thyroxine + testosterone	♂	14	332	5.4	> 5.9	.05	.002
	♀	15	261	6.4			
I^{131} + thyroxine	♂	18	340	7.4	> 7.5	.11	.01
	♀	19	229	7.6			
I^{131} + testosterone	♂	15	201	11.5	>11.6	.01	.05
	♀	17	178	11.7			
I^{131} + thyroxine + testosterone	♂	15	331	8.6	> 8.3	<.5	.02
	♀	16	252	8.0			

* Probability was calculated from No. of lesions with sexes combined, and the probability values should be compared only within each column.

ectomized rats to that of the control value by replacing the thyroid hormone. Thyroxine administered subcutaneously was used for this purpose instead of desiccated thyroid in the diet as used in previous studies since by use of injection technics it is possible to accurately regulate the dosage. Inasmuch as testosterone has been shown to be necessary for the histological maintenance of the submaxillary gland(6), it was also included in these studies.

Materials and methods. A total of 280 weanling Sprague-Dawley strain rats were divided into 7 experimental groups according to sex and initial body weight. Four groups received 500 μc of carrier-free I^{131} by subcutaneous injection; the first of these groups received no hormone supplement while the second, third and fourth groups received respectively 50 μg of thyroxine daily by subcutaneous injection, 2.5 mg/week/animal of testosterone propionate in croton oil injected intra-muscularly and a combination of thyroxine and testosterone at the same levels as used above. Two non-thyroidectomized groups received hormone injections, one receiving thyroxine and the other testosterone at the same levels as used in the radiothyroidectomized rats, while a final group received no radioiodine or hormone supplements and served as a control group. All of the animals were housed in raised screen cages and received the same cariogenic stock corn diet† (Fluorine = 0.5 μg/g) and fluorine-low (F = 0.5 μg/ml) drinking water. The duration of the experiment was 140 days after which time the animals were sacrificed by ether, the heads removed for dental caries evaluation by methods previously described(7) and the salivary glands and thyroid glands fixed for histologic study.

Results. The results of these experiments are seen in Table I. The injection of radioiodine results in a significant increase in the incidence of dental caries and confirms previous studies(3). Similarly, when thyroxine was injected into non-thyroidectomized rats the incidence of dental caries was significantly decreased. When both thyroxine and testosterone were injected into non-thyroidectomized rats the dental caries incidence was essentially the same as the non-thyroidectomized group which received thyroxine.

The injection of thyroxine into thyroidectomized rats produces a significant decrease in the caries rate and results in the same incidence of dental caries as found in the control group. Testosterone injection does not decrease the caries incidence to the same degree as thyroxine in thyroidectomized rats, but does result in significantly less dental caries than found in the non-hormone injected radiothyroidectomized animals. The injection of both thyroxine and testosterone into radiothyroidectomized rats results in approximately the same incidence in caries as found in the controls.

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† The composition of diet used was as follows: yellow corn grits, 52.7%; ground yellow whole corn, 11.3%; powdered whole milk, 30.0%; alfalfa, 4.8%; iodized sodium chloride, 1.0% and irradiated yeast, 0.2%.

The thyroid glands of the animals which received radioactive iodine exhibited the expected microscopic findings of severe fibrosis with loss of recognizable follicles in most instances. The submaxillary salivary glands reacted to the thyroid disturbance in a manner similar to that following the administration of propylthiouracil. The granular tubules were markedly diminished in size and exhibited severe loss of granules in those animals which had been radiothyroidectomized. The administration of thyroxine alone or in combination with testosterone to radiothyroidectomized rats returned the structure of the submaxillary glands to that comparable to the control animals. Testosterone alone did not exhibit this ability. It may be concluded, therefore, that thyroxine itself or one of its metabolic products is responsible for maintenance of the submaxillary gland granular tubules, directly or indirectly.

Discussion. The relationship between the thyroid gland and the dental caries incidence is clearly shown in this study. When thyroxine is injected into non-thyroidectomized rats a significant decrease in the caries incidence results while radioiodide causes a significant increase in the incidence of dental caries. Thus, the amount of thyroxine used in this study and desiccated thyroid fed as a component of the diet used in previous studies appear to have about the same degree of anticariogenicity. The simultaneous administration of thyroxine and testosterone does not appear to have a synergistic effect on caries, and results in about the same decrease in dental caries as found by the injection of thyroxine alone.

The marked anticariogenic effect of thyroxine is best manifested in the radiothyroidectomized group. In this instance the administration of thyroxine results in a marked decrease in the dental caries experience. Testosterone also significantly decreases the caries experience in radiothyroidectomized rats, suggesting that both of these hormones are necessary for maintaining the normal salivary gland function in the rat. These studies seem to convincingly implicate the thyroid hormone in maintaining the function of the salivary glands and in turn directly affecting the incidence of dental caries in the rat.

Summary. The activity of the thyroid gland appears to be directly related to the incidence of dental caries in the rat, for thyroxine injected significantly decreases the caries rate and radiothyroidectomy significantly increases it. The thyroid hormone appears to be primarily involved since the increased caries rate resulting from radiothyroidectomy is directly counteracted by the administration of thyroxine. Testosterone similarly appears to play a role in this mechanism, since the dental caries rate is also significantly reduced when it is injected into radiothyroidectomized animals even in the absence of thyroxine.

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1. Muhler, J. C., and Shafer, W. G., *Science*, 1954, v119, 687.

2. ———, PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 191.

3. Bixler, D., Muhler, J. C., and Shafer, W. G., *J. Am. Dent. Assn.*, in press.

4. Shafer, W. G., and Muhler, J. C., *J. Dent. Res.*, 1955, v34, 531.

5. Kochakian, C. D., Endahl, B. R., and Hall, H. D., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 289.

6. Grad, B., and Leblond, C. P., *Endocrinology*, 1949, v45, 250.

7. Muhler, J. C., and Day, H. G., *J. Am. Dent. Assn.*, 1950, v41, 528.

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Effects of Penicillin G and Penicillin V on Thiamine Synthesis in the Rat.*

(22747)

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A few workers have related the growth promoting ability of antibiotics to elevated levels of certain limiting dietary vitamins in tissues and/or intestinal ingesta(1-6). In this study, an attempt has been made to correlate hepatic and intestinal levels of thiamine with the rate of growth of young rats fed rations containing a sub-optimal concentration of vit. B_1 and supplemented with one of three salts of penicillin G (benzyl penicillin) or with sodium penicillin V (phenoxymethyl penicillin).

Materials and methods. Forty weanling Sprague-Dawley albino female rats of between 40 and 50 g in weight were fed a semi-purified, thiamine-inadequate diet of the following percentage composition: "vitamin-free" casein (Nutritional Biochem. Corp.) 20, dextrinized corn starch 28, sucrose 39, hydrogenated vegetable oil (Crisco) 2.8, corn oil (Mazola) 4, salts (U.S.P. XIV) 4, L-cystine 0.2, and powdered cellulose (Alphacel, Nutritional Biochem. Corp.) 2. The following vitamins were added to the ration (mg/kg): thiamine hydrochloride 0.4, riboflavin 7.0, pyridoxine hydrochloride 5.0, calcium pantothenate 25, nicotinamide 25, i-inositol 100, choline chloride 1,000, folic acid 0.5, biotin 0.1, *para*-aminobenzoic acid 25, 2 methyl-1, 4-naphthoquinone 2.5, and vit. B_{12} 0.025. The corn oil additive contained 0.5 g oleum percomorphum and 125 mg d-alpha tocopherol/100 g. A portion of the carbohydrate component of this ration was in the form of dextrin because Sauberlich(7) reported antibiotics to overcome more readily a thiamine inadequacy if the dietary carbohydrate were dextrin than if it were sucrose. After a 5-day depletion period, groups of 4 animals were subjected to the following regimen:

I	Basal ration
II	*Idem* + potassium penicillin G
III	” + procaine penicillin G
IV	” + benzathine penicillin G
V	” + potassium penicillin V
VI	” + 5.6 mg thiamine hydrochloride/kg
VII	” + injections of potassium penicillin G
VIII	” + ” ” procaine penicillin G
IX	” + ” ” benzathine penicillin G
X	” + ” ” potassium penicillin V

Penicillin was incorporated in the diets at a level of 100 mg/kg ration or injected intraperitoneally (0.5 mg) once every 48 hours. Food and water were provided as needed and the animals were weighed at regular intervals. No record was kept of the amount of food consumed. On the 27th day of treatment, groups I-V were killed by a sharp blow on the head and their intestines and livers excised. The contents of each ileum and cecum were expelled into small screw-cap jars and frozen immediately in a dry ice chest. Each liver was weighed and also frozen. Each of the 3 types of specimens was pooled by ration group. On the 28th day the remaining animals (Groups VI-X) were killed and livers and intestinal contents recovered as described for Groups I-V. The thiamine content of each sample was determined subsequently by means of a microbiologic assay using *Lactobacillus fermenti* 36(8). The livers were thawed and dispersed in water with a Potter-Elvehjem homogenizer prior to assaying them for thiamine. The intestinal samples were thawed as needed, suitably diluted in water, mixed thoroughly and a portion centrifuged at 2,500 rpm in a Serval model SP/X centrifuge. The supernatant liquid was recovered and assayed directly while the portion of the suspension which had not been centrifuged was prepared for assay in the recommended manner by over-night digestion with pepsin and takadiastase. Thus, the intestinal contents were assayed for "free" and "total" thiamine. Just "total" thiamine was estimated from assays of the liver preparations.

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TABLE I. Effects of Oral and Parenteral Administration of 3 Different Salts of Penicillin G and One of Penicillin V on the Growth of Weanling Rats Fed a Thiamine-Inadequate Diet. Average 27-day net gain in body wt (g) ± stand. dev.

Treatment	Mode of administration of penicillin Oral*	 Parenteral†
K penicillin G (II & VII)	98.38 ± 7.57	88.25 ± 22.10
Procaine penicillin G (III & VIII)	91.13 ± 10.66	75.25 ± 15.99
Benzathine penicillin G (IV & IX)	99.00 ± 7.60	61.50 ± 14.72
K penicillin V (V & X)	88.25 ± 11.21	73.25 ± 11.62
Thiamine-insufficient control (I)	50.75 ± 21.20	
Thiamine-sufficient " (VI)	105.63 ± 7.14	

* By means of analysis of variance of these data (Group VI excluded from analysis) a highly significant difference exists. LSD$_{.01}$ = 26.4.

† No statistically significant difference was found in these groups. Calculated as indicated in footnote *.

Results. The growth data assembled in Table I reveal that: a) the level of thiamine employed in the basal ration was not adequate for normal growth; b) supplementation of the deficient basal ration with excess thiamine resulted in normal growth rates; c) ingestion of penicillin stimulated growth significantly (indicative of an anticipated thiamine-sparing action); and d) intraperitoneal administration of the penicillins was not accompanied by a statistically-significant growth response. It is of interest that the three salts of penicillin G and the one of penicillin V were essentially equal in their ability to spare the dietary requirement for thiamine when given *per os*. It would appear that potassium penicillin G was superior to the other penicillins when administered parenterally, although the growth response was not significant when subjected to an analysis of variance.

The results of the thiamine assays of the livers are seen in Table II. Note the consistently higher concentrations of hepatic thiamine in rats given penicillin than in the deficient control rats (Group I). It is apparent also that the oral route of administration of penicillin was more effective than the parenteral one in enhancing storage of thiamine in the liver. When expressed as the amount of thiamine per liver, the stimulatory action of penicillin on the hepatic stores of vit. B_1 is even more striking than an expression of concentration. Hence, penicillin had an effect upon the size of the liver in these rats. Also, it would seem that potassium penicillin G was more effective in encouraging liver stores of thiamine than the other penicillins tested.

The data in Table III are not particularly illuminating in suggesting reasons for effects of penicillin on liver stores of thiamine. Note that in the ileum both "free" and "total" thia-

TABLE II. Effect of Penicillin on Weights and Thiamine Levels of Livers from Rats Fed a Thiamine-Inadequate Ration. Expressed as fresh wt of liver.

Treatment		Avg thiamine content Conc. (μg/g)	 Total (μg/liver)	Avg liver wt (g)
Thiamine-insufficient control		.84	4.27	5.11 ± 1.12*
Thiamine-sufficient "		2.82	21.09	7.48 ± 1.53
K penicillin G	Oral	1.54	10.76	7.01 ± .29
	Parenteral	1.64	9.93	6.04 ± 1.47
Procaine penicillin G	Oral	1.29	8.98	6.96 ± 1.21
	Parenteral	1.07	5.56	5.18 ± .96
Benzathine penicillin G	Oral	1.21	8.99	7.45 ± 1.11
	Parenteral	1.06	5.33	5.03 ± 1.17
K penicillin V	Oral	1.22	8.44	6.93 ± .91
	Parenteral	1.06	5.77	5.45 ± 1.08

* ± stand. dev.

TABLE III. Thiamine Levels in Ileal and Cecal Contents of Rats Given Penicillin and a Thiamine-Inadequate Diet. Results expressed as μg/g wet wt.

Treatment		Ileum		Cecum	
		"Free"	"Total"	"Free"	"Total"
Thiamine-insufficient control		.34	.70	.38	1.00
Thiamine-sufficient "		.90	1.69	.63	2.68
K penicillin G	Oral	.17	1.57	.18	.76
	Parenteral	.005	.51	.31	1.37
Procaine penicillin G	Oral	.45	1.53	.03	.53
	Parenteral	.02	.52	.91	
Benzathine penicillin G	Oral	.19	.64	.30	.51
	Parenteral	.005	.47	.69	1.49
K penicillin V	Oral	.17	.60	.45	.85
	Parenteral	.005	.005	.83	1.47

mine existed at greater concentrations in animals given the antibiotic orally than in those which had received penicillin intraperitoneally. However, in no case was the concentration of "free" thiamine in ileal contents increased by the administration of penicillin; in only two cases (orally-administered potassium and procaine penicillin G) did penicillin raise the level of "total" thiamine in ileal ingesta. The only consistent pattern in cecal contents is that parenteral administration of penicillin, with but one exception, elevated the levels of "free" and "total" thiamine while oral administration of the drug had an opposite effect.

Discussion. This report adds weight to the accumulating evidence that antibiotics aid in the vitamin economy of certain animals. However, the manner by which this is achieved is unknown. True, the intestinal flora is affected(3,5,6), a fact which we felt unnecessary to establish in this report. Moreover, absorption from the intestinal lumen should be considered in view of reports that antibiotics cause a thinning of the intestinal wall(9,10). Unfortunately, our data on the influence of penicillin on the thiamine content of the intestinal ingesta are inconclusive. Nevertheless, the positive effect of penicillin on liver stores of thiamine may be a reflection of intestinal synthesis and absorption, a supposition advocated by others(2,4,9).

Apparently the different physical properties of the penicillin salts tested had little to do with the thiamine-sparing activity of penicillin, since the 3 salts of penicillin G and the one salt of penicillin V differed but slightly in their effects on the rate of growth. It is obvious further that incorporation of the antibiotic in the ration gave a growth response superior to that obtained with widely-spaced parenteral injections of penicillin. It must be remembered, however, that all of the penicillin given parenterally is excreted within 6 to 8 hours; thus the tissues of rats which received penicillin parenterally were exposed to penicillin approximately 8 of the 48 hours that elapsed between injections. On the other hand, bacitracin, an antibiotic that is poorly absorbed from the intestinal tract, has been reported to stimulate the growth of rats fed a vit. B_{12} deficient diet only when administered intraperitoneally(11). Presumably, if penicillin had been given to the rats parenterally every 8 hours instead of every 48 hours a growth response may have occurred. Despite the long interval between injections of penicillin, the total amount and concentration of thiamine in the livers of these animals was appreciably greater than in the thiamine-deficient control animals. Moreover, of the parenterally administered penicillins, potassium penicillin G enhanced the rate of growth as well as the liver's stores of thiamine to the greatest degree. Correlation of the growth rates with the content of thiamine in the livers and the liver weights is excellent (compare Tables I and II). Nevertheless, the thiamine-sufficient control rats had approximately twice the amount of thiamine in their livers than did the deficient rats showing the best response to penicillin.

Summary. Three salts of penicillin G and one of penicillin V were found to be essentially equal when administered orally in sparing the rat's dietary requirement for thiamine, as determined by rates of growth and thiamine levels in the liver. Growth was not stimulated to a statistically significant degree upon administering these antibiotics intraperitoneally every other day, although thiamine levels in the livers were elevated by such treatment. The effects of penicillin on thiamine levels in ileal and cecal contents were inconclusive.

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1. Lih, H., and Baumann, C. A., *J. Nutrition,* 1951, v45, 143.

2. Guggenheim, K., Halevy, S., Hartmann, I., and Zamir, R., *ibid.,* 1953, v50, 245.

3. Peterson, G. E., Dick, E. C., and Johansson, K. R., *ibid.,* 1953, v51, 171.

4. Monson, W. J., Harper, A. E., Winje, M. E., Elvehjem, C. A., Rhodes, R. A., and Sarles, W. B., *ibid.,* 1954, v52, 627.

5. Jukes, T. H., *Antibiotics in Nutrition,* Med. Encycl., New York City, 1955.

6. Johansson, K. R., *Proc. 1st Int. Conf. Antibiotics Agr.,* Publ. 397, Nat. Acad. Sciences, Nat. Research Council, 1956, 127.

7. Sauberlich, H. E., *J. Nutrition,* 1952, v46, 99.

8. Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.,* 1944, v155, 153.

9. Baumann, C. A., *Proc. 1st. Int. Conf. Antibiotics Agr.,* Publ. 397, Nat. Acad. Sciences, Nat. Research Counc., 1956, 47.

10. Braude, R., Coates, M. E., Davies, M. K., Harrison, G. F., and Mitchell, K. G., *Brit. J. Nutrition,* 1955, v9, 363.

11. Peterson, G. E., and Johansson, K. R., *Antibiotics Annual* 1953-1954, Med. Encycl., New York City, 1953, 386.

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Effect of Stimulation of Adrenal Cortex on Blood Pepsinogen in Animals and Man. (22748)

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The level of pepsinogen in blood and urine constitutes an index of gastric activity(1-4). Uropepsinogen excretion reportedly increases following administration of adrenocorticotrophic hormone or adrenal steroids, and after acute emotional and physical stress; therefore it has been suggested as a measurement of adrenal activity. These data and their implications concerning the relationship of gastric activity to stress and the General Adaptation Syndrome have been reviewed recently by Gray *et al.*(5). However, Hirschowitz and his associates were not able to demonstrate increases in blood pepsinogen following ACTH administration(6), and Necheles and his co-workers recently reported failure of cortisone to affect uropepsinogen excretion (7). Similarly, in animal studies, we were unable to demonstrate elevation in blood pepsinogen following either acute or chronic noxious stimulation in cats and rats even when adrenal hypertrophy was observed(8,9). A study was undertaken, therefore, to determine if direct stimulation of the adrenals by exogenous ACTH influenced blood pepsinogen in animals. The effect of a single injection of ACTH on blood pepsinogen in humans was also evaluated and titres in several patients on chronic steroid therapy were measured.

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TABLE I. Response of Blood Pepsinogen to Acute Adrenal Stimulation in Animals.

		Blood pepsinogen activity		
Animal	Treatment	Before	After	Hr after inj.
Cats A	Saline	290	260	24
B	50 units ACTH gel	375	315	24
C	*Idem*	310	335	24
Dogs A	Saline	240	250	6
			260	24
B	40 units ACTH gel	490	350	6
			410	24
C	*Idem*	355	400	6
			405	24
Rats 1	Saline	260	275	24
2	"	250	205	24
3	10 units ACTH gel	265	320	24
4	*Idem*	—	210	24
5	Saline	260	245	48
6	"	285	375	48
7	"	300	255	48
8	30 units ACTH gel	—	325	48
9	*Idem*	320	240	48
10	"	345	340	48

Materials and methods. Acute studies were performed in 3 mongrel cats, 3 mongrel dogs, and 10 male Holtzman rats in whom blood pepsinogen was determined before and after administration of ACTH gel. Treatments and time of measurement of pepsinogen are given in Table I. Acute studies were also performed in 9 healthy human subjects, one patient with pernicious anemia and one with renal disease. In the latter condition, pepsinogen activity may be elevated, as has been demonstrated by Hirschowitz(3) and Spiro (10). In addition, pepsinogen was determined following chronic therapy in one patient with an inactive peptic ulcer and severe pulmonary disease, and in four patients without ulcers, who were receiving large doses of steroids and/or ACTH for a variety of ailments. Diagnoses, dosage schedules and experimental design in the human subjects are presented in Table II. To study adrenal cortical activity and blood pepsinogen levels simultaneously, 42 male Holtzman rats weighing 200-250 g were divided randomly into 4 groups. Blood was obtained by cardiac puncture, and the animals were treated for 5 days as outlined in Table III. All animals were then anesthetized with sodium pentobarbital and rapidly exsanguinated. Adrenals were cleaned of extraneous tissue, weighed to 0.1 mg on a Sartorius balance, and their ascorbic acid content determined by the method of Roe(11). Blood pepsinogen activity was determined by the method of Mirsky *et al.*(12). One unit of pepsinogen represents one microgram of tyrosine released by one ml of plasma or serum; plasma was used for the cats and dogs, serum in the rats and humans.

Results. A consistent increase in pepsinogen activity was not noted in the acute studies (Tables I and II). In 4 patients who were receiving chronic treatment with steroids, blood pepsinogen was within the normal range in our laboratory (*v.i.*) and rose slightly in only one of 3 with further treatment. Activity increased in the ulcer patient after one week of therapy with prednisone alone, but thereafter fell to initial levels despite continued administration of ACTH and prednisone (Table II). The effects of adrenal cortical stimulation on adrenal weight, ascorbic acid content and level of pepsinogen in rats are set forth in Table III. These data were subjected to an Analysis of Variance. The adrenals of the groups which received ACTH and epinephrine were significantly heavier and demonstrated a highly significant depletion of ascorbic acid, but pepsinogen activity was identical in all groups. Comparison of the pepsinogen values before and after treatment in each of the groups suggested a slight fall in the groups given ACTH and epinephrine, but this was not significant statistically. Carbaminoylcholine (Doryl), a parasympathomimetic agent with predominant gastro-intestinal effects, did not stimulate the adrenal nor elevate the average pepsinogen level; however 2 animals in the group displayed titres which were over 500 units. Peptic ulceration was not observed.

Serum pepsinogen levels determined in our laboratory in 18 normal patients ranged from 195 to 445 with a mean of 305 units, and a standard deviation of 78.5. In 24 patients with proven peptic ulcer, the values ranged from 366 to 968, with a mean of 610 and standard deviation of 162. Although there is

TABLE II. Response of Blood Pepsinogen to Adrenal Stimulation in Human Subjects.

Acute studies (11 subjects)

Sex	Treatment (ACTH gel IM), units	Blood pepsinogen activity		Sex	Treatment (ACTH gel IM), units	Blood pepsinogen activity	
		Before	24 hr after			Before	24 hr after
♀	40	300	340	♂	40	435	530
♂	40	295	300	♂	40	280	285
♂	40	290	240	♀	40	205	200
♂	40	205	290	♂*	40‡	50	60
♀	40‡	305	300	♀†	40‡	735	440
♂	40	250	210				

* Patient with pernicious anemia. † Patient with nephrotic syndrome and blood urea nitrogen of 50 mg %. ‡ Aqueous ACTH IM.

Chronic studies (5 patients)

Diagnosis	Treatment	Pepsinogen activity	Time (day of study)
Inactive peptic ulcer; chronic pulmonary disease	(1) Prednisone daily from day 0 to 60 (total dosage 490 mg)	890	0
		1150	8
		900	16
	(2) ACTH gel IM twice weekly from day 17 through 60 (total dose 260 units)	955	23
		905	30
		905	60
Drug sensitivity	(1) 5 to 10 mg Prednisone daily for 3 mo prior to day 0	400	0
	(2) 40 units ACTH gel IM daily from day 0 to 4	495	4
	(3) No therapy; day 4 to day 8	425	8
Undiagnosed collagen disease	15 mg Prednisone daily for 13 days prior to day 0 (total dosage 195 mg)	275	0
Rheumatoid arthritis, drug-induced Cushing's syndrome with marked hypertension	(1) Cortisone 25 mg daily for 20 days every mo for 3 yr prior to day 0	390	2
	(2) 40 units ACTH gel IM day 0 to day 7; 25 mg cortisone day 0 to day 2; 12.5 mg daily days 3 and 4	220	6
Drug sensitivity	(1) 40 units ACTH gel IM and 10 mg Prednisone; day 0	193	3
	(2) 40 units aqueous ACTH I.V.; day 1 and 2		

TABLE III. Comparison of Blood Pepsinogen Activity and Adrenal Cortical Activity in Rats.

Treatment*	No. of rats	Means and stand. dev.			
		Adrenal wt (mg/100 g body wt)	Adrenal ascorbic acid (mg/100 g adrenal)	Pepsinogen activity (μg tyrosine released/cc serum)	
				Before	After
Control	10	16.7 ± 2.1	455 ± 26.9	256 ± 79	251 ± 163
ACTH	11	22.6 ± 3.7	333 ± 53.9	264 ± 75	210 ± 99
Doryl	11	15.3 ± 1.7	462 ± 41.6	236 ± 85	264 ± 168
Epinephrine	10	19.8 ± 1.8	367 ± 38.9	257 ± 51	198 ± 75
p values Between groups		<.01	<.01	N.S.	N.S.†
Control × ACTH		.001	.001	Grand mean (before) 253 ± 74	
Control × doryl		N.S.	N.S.		
Control × epinephrine		.04	.001		

* Dosage schedules (all treatments of 5 days duration):

Control, 1 ml normal saline daily; ACTH, 10 units of ACTH gel daily; Doryl, 1.5 μg twice daily; Epinephrine, 0.4 mg (in oil) daily.

† Differences between grand mean before treatment and means of individual groups after treatment, including apparent declines in ACTH and epinephrine groups, similarly are not significant.

an overlap in the range from 350 to 450, the differences between means is highly significant ($p = .001$). In 5 patients with pernicious anemia the mean value was 137. The patients in these 3 groups all had normal levels of blood urea nitrogen. In 9 patients with blood urea nitrogen over 20 mg % (range 20 to 135), pepsinogen levels averaged 615 with standard deviation of 213. These data are reported to add our results to the literature confirming the significant elevation of blood pepsinogen in peptic ulcer, its depression in pernicious anemia, and its increase in renal failure.

Discussion. Failure to produce an increase in blood pepsinogen consistently following acute administration of ACTH indicates that the level of this enzyme cannot be considered a sensitive and reliable index of adrenal cortical activation. The studies in rats in which pepsinogen and adrenal cortical activity were measured simultaneously clearly indicate that stimulation of the adrenals sufficiently intense to cause hypertrophy and depletion of ascorbic acid, will not raise blood pepsinogen and may perhaps lower it. Nor was an appreciable elevation of blood pepsinogen noted in human subjects receiving chronic or large doses of ACTH and/or steroids. Accordingly, an elevated level of blood pepsinogen need not indicate adrenal hyperactivity, nor should it be interpreted as evidence that the General Adaptation Syndrome is causally linked to the development of peptic ulcer. These considerations do not obviate the fact that prolonged administration of ACTH or cortical steroids may produce an acute ulcer in some patients, after which pepsinogen may rise(6). However, in such instances the adrenal hormone probably plays a permissive, rather than a causal, role and operates by increasing the sensitivity of the gut to stimuli previously not damaging(13). Direct tissue changes, vascular disturbances, or changes in the character of the mucous secretion of the stomach represent possible mechanisms for this action, which need not then be associated with a prior rise in pepsin secretion.

The increase in *urinary* pepsinogen following ACTH or cortical steroids reported by Gray(5) is not explained by our data. Hirschowitz has demonstrated that gastric pepsin in the human does not increase regularly after ACTH while blood pepsinogen is essentially unchanged, and suggests that the increased urinary output reflects increased renal clearance and not increased production(6). These circumstances should of course result in an actual fall in blood pepsinogen, which is suggested by our data in rats and in the patient with impaired renal function whose blood level declined after ACTH. Accurate delineation of this problem however requires simultaneous measurement of urine, blood, and gastric pepsin under a variety of conditions. The precise meaning of changes in levels in blood and urine might still be obscured because the volume of blood considerably dilutes the small quantities of pepsinogen available for renal clearance, and the methods for measurement carry an inherent error of 5 to 10%. Accordingly, until these arguments are clarified, it would seem well advised to consider blood pepsinogen only as an indicator of gastric activity which is fairly constant in most individuals, elevated in patients with peptic ulcer, and not influenced by acutely stressful situations. Changes which occur in chronic situations with deep-seated psychodynamic dislocations as described by Mirsky(14), probably have no direct relationship to adrenocortical stimulation.

Summary. Blood pepsinogen levels were measured in cats, dogs, rats, and man following acute and chronic administration of adrenocorticotrophic hormone and/or cortical steroids. Despite demonstration of adrenal cortical stimulation, pepsinogen activity did not increase. The data indicate that blood pepsinogen activity does not constitute an index of adrenal cortical activation.

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1. Podore, C. J., Broh-Kahn, R. H., and Mirsky, I. A., *J. Clin. Invest.*, 1948, v27, 834.

2. Mirsky, I. A., Futterman, P., and Kaplan, S., *J. Lab. and Clin. Med.*, 1952, v40, 188.

3. Hirschowitz, B. I., *ibid.*, 1955, v46, 568.

4. Spiro, H. M., Ryan, A. E., and Jones, C. M., *New Eng. J. Med.*, 1955, v253, 261.

5. Gray, S. J., Ramsey, C. G., Villarreal, R., and Krakauer, L. J., in *Fifth Annual Report on Stress,*

edited by Selye, H., and Heuser, G. M. D. Publications, Inc., New York City, 1956.

6. Hirschowitz, B. I., Streetin, D. H. P., Pollard, H. M., and Boldt, H. A., Jr., *J.A.M.A.*, 1955, v158, 27.

7. Necheles, H., Myer, J., Bridgewater, A. B., Sorter, H., and Wulkan, E., *J. Appl. Physiol.*, 1956, v8, 559.

8. Shapiro, A. P., and Horn, P. W., *J. Nerv. and Ment. Disease*, 1955, v122, 222.

9. Belkin, G. W., and Shapiro, A. P., *Texas Rep. Biol. and Med.*

10. Spiro, H. M., Ryan, A. E., and Jones, C. M., *Gastroenterology*, 1956, v30, 563.

11. Roe, J., *Biochemical Analysis*, Vol. 1, Interscience Publishers, New York City, 1954, p127.

12. Mirsky, I. A., Futterman, P., Kaplan, S., and Broh-Kahn, R. H., *J. Lab. and Clin. Med.*, 1952, v40, 17.

13. Engel, F. L., *J. Clin. Endocrin.*, 1955, v15, 1300.

14. Mirsky, I. A., Kaplan, S., and Broh-Kahn, R. H., *Proc. A. R. Nerv. and Ment. Dis.*, 1950, v29, 628.

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Chromatographic Separation of Growth Hormone and Other Peptides.*
(22749)

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A chromatographic system was devised for the purification of growth hormone which appears, in addition, to be generally useful for the fractionation of polypeptides. The system may be adapted to either of two means of separation. The difference in maximal acetic acid concentration allowing adsorption on carboxymethylcellulose is sufficient to permit separation of some substances. Fractions which have similar adsorbability from acetic acid are separated by elution from carboxymethylcellulose with weak solutions of neutral salts in acetic acid. The resolving power of the salt elution appears to be particularly high when the acetic acid concentration is rather near the maximum strength permitting adsorption. The appropriate acetic acid concentration may be predetermined by small batch experiments, or by gradient elution from a column with the acid.

Results. Experiments with a purified growth hormone preparation indicated that adsorption occurred in acetic acid in strengths up to approximately 3.5 M. Gradient elution with acetic acid separated a minor component, but most of the material appeared with a sharp front at about 3.6 M but with extensive tailing and no further fractionation. Gradient elution with calcium chloride in 1.0 M acetic acid eliminated the tailing and yielded

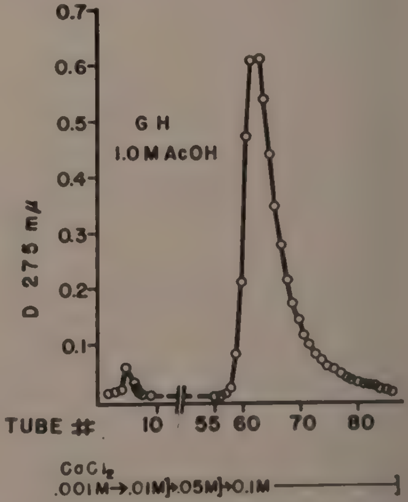

FIG. 1. 30 mg pig growth hormone preparation in 1.0 M acetic acid containing .001 M $CaCl_2$ on 3.5 g carboxymethylcellulose prewashed with same solution and packed under pressure. Column dimensions: 9 × 220 mm. Mixing flask for the gradient elution contained a constant vol of 120 cc. Flow rate, 7.4 ml/hr.

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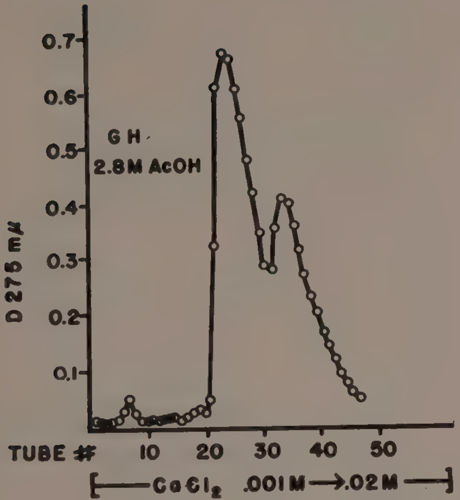

FIG. 2. Same as Fig. 1, except for changes in acetic acid and calcium chloride concentrations. Flow rate, 5.2 ml/hr.

one minor and one major fraction (Fig. 1); elution with calcium chloride in 2.0 M acetic acid separated the major fraction into two components, and the separation was somewhat better with 2.8 M acid (Fig. 2). The growth-promoting activity was concentrated in the faster running of the two components.

There was an inverse relation between acetic acid concentration and the concentration of salt required for elution. Elution with lanthanum chloride rather than calcium chloride decreased the required molar concentration of salt by about one-half. Gradient elution with calcium chloride in 0.3 M formic acid failed to separate the two major components, suggesting that the solvent character of the strong acetic acid solutions may contribute to the fractionating power of the system. However, the addition of .01 M calcium acetate to acetic acid prevented separation of the major components by gradient elution with calcium chloride, presumably due to the buffering effect. In general, resolution appeared to be improved by conditions favoring a weakened affinity of the adsorber for the peptide, and this may account for the impression obtained by Ellis and Simpson(1) that the resolving power of carboxymethylcellulose for growth hormone preparations was not particularly high under their conditions which were chosen to favor maximum adsorption.

Little, if any, growth hormone was bound irreversibly by the adsorbent, but loss of other protein material to the column may occur. The recovery from these columns was about 80%, but with another growth hormone preparation (Armour R285-128) slightly less than half the charge emerged under the same conditions. Application of a larger charge of the Armour material on a fresh column or reuse of the same column for the same charge yielded a major fraction of growth-inactive material appearing with the front and a growth-active fraction in the anticipated position.

Applying the information obtained from these studies, it was found possible to prepare growth hormone directly from a crude pituitary extract either by gradient elution, or by fractional elution with fixed concentrations of calcium chloride in 2.0 M acetic acid.

A purified corticotropin preparation was not adsorbed by carboxymethylcellulose from a 2.0 M solution acetic acid, but considerable fractionation was obtained by gradient elution with calcium chloride in a 1.0 M acetic acid solution (Fig. 3). Intermedin activity was concentrated in the third fraction and corticotropin activity in the unresolved fourth and fifth fractions. The small amount of spermine present in the preparation(2) was concentrated in the region of the second peak,

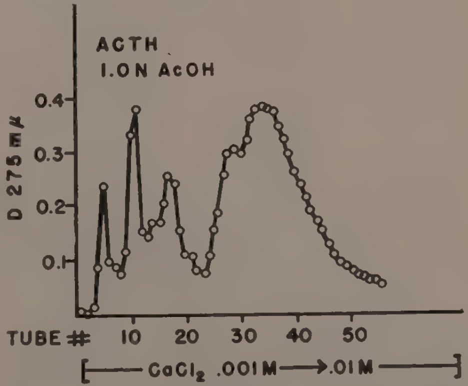

FIG. 3. 24 mg oxycel-purified corticotropin on column similar to those of Fig. 1 and 2. Flow rate, 6.4 ml/hr.

as judged by the formation of characteristic picrate crystals.

The carboxymethylcellulose used in these experiments was prepared from Solka-Floc BW according to the directions of Peterson and Sober(3) and contained approximately 0.75 milliequivalent carboxyl group per gram. The growth hormone was prepared from pig pituitaries as previously described(4), and the corticotropin preparation was purified by adsorption on oxidized cellulose as described by Astwood *et al.*(5).

It is of interest that carboxymethylcellulose adsorbed practically none of the corticotropin preparation from 2.0 M acetic acid but adsorbed 68 mg/g of the growth hormone preparation, since it was previously noted that 10.4% carboxyl oxidized cellulose adsorbed, from 0.1 M acetic acid, 340 mg/g of the corticotropin and only 6 mg/g of growth hormone(4).

Summary. A chromatographic system of high resolving power was devised for the purification of growth hormone and was adapted for use in the separation of other peptides.

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1. Ellis, S., and Simpson, M. E., *J. Biol. Chem.*, 1956, v220, 939.

2. Astwood, E. B., Raben, M. S., Rosenberg, I. N., and Westermeyer, V. W., *Science*, 1953, v118, 567.

3. Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, 1956, v78, 751.

4. Raben, M. S., and Westermeyer, V. W., Proc. Soc. Exp. Biol. and Med., 1951, v78, 550.

5. Astwood, E. B., Raben, M. S., Payne, R. W., and Grady, A., *J. Am. Chem. Soc.*, 1951, v73, 2969.

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Effect of Allergic Shock on Fate of Staphylococci in the Organs of Mice. (22750)

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As recently shown by several investigators (1,2,3) an anaphylactic type of sensitization can be elicited in mice by injecting intraperitoneally certain protein antigens in association with pertussis vaccine. The preliminary results here reported indicate that a mild allergic shock induced in animals so sensitized can modify their resistance to bacterial infections.

Experimental. Specific sensitization of mice to bovine serum. The ability of mice to develop an anaphylactic type of sensitization to bovine serum is illustrated in the following experiments with two different breeds of mice. Female mice of so-called Rockefeller Swiss strain were used when 7 weeks old; their average weight was 31 g. They were fed bread and milk throughout the experiment and sensitized as indicated in Table I. The bovine serum was a sterile filtrate distributed by Difco laboratories. The pertussis vaccine was a preparation distributed for human use by Lederle Laboratories and obtained through the kindness of Dr. Stanton M. Hardy. It contained approximately 60 billion bacterial cells/ml. The animals were challenged in groups of 5 by intravenous injection of various amounts of serum. All deaths occurred within 30-60 minutes (Table I).

Irrespective of the amount of serum used for the challenge injection, there were no deaths among the mice pretreated with either physiological saline, or with pertussis vaccine alone (Table I). In contrast, many of the animals that had been pretreated with serum died shortly after the challenge injection, even when the challenge dose was smaller than 0.01 ml. There was evidence furthermore that the degree of sensitization had been increased by injecting pertussis vaccine in addition to bovine serum at the time of sensitization.

The following experiment carried out with another strain of mice provides evidence that

TABLE I. Effect of Pertussis Vaccine on Sensitization of Mice* to Bovine Serum.

Sensitizing treatment (i.p.)		Challenge† (i.v.) ml serum	Deaths‡ (out of 5)
Serum	Pertussis vaccine		
ml	ml		
.5	.05	.1	5
		.01	5
		.003	2
		.001	3
.5	0	.1	3
		.01	2
		.003	1
0	.05	.1	0
		.01	0
Saline controls		.1	0
		.01	0

* Female Rockefeller Swiss mice, 7 wk old.
† Three wk after sensitization.
‡ All deaths occurred within 30-60 min.

the allergic state elicited by serum-pertussis mixtures is specific. Female mice of the CF_1 breed were obtained from Carworth Farms and maintained on a diet of bread and milk. Individual weights ranged from 20-28 g when they received the sensitizing pretreatment. The bovine serum and pertussis vaccine were the same preparations that had been used in the preceding experiment. Fourteen days or 19 days after pretreatment, the animals were challenged with various amounts of bovine serum, rabbit serum, or egg albumin, in a final volume of 0.2 ml (Table II).

The results presented in Table II show that following treatment with bovine serum and pertussis vaccine, CF_1 mice developed a degree of sensitization comparable to that developed by mice of the Rockefeller Swiss breed. With both breeds of mice, intravenous injection of an amount of serum as small as 0.001 ml administered approximately 3 weeks after sensitizing pretreatment was sufficient to cause acute anaphylactic-like death (within 30-60 minutes) of a certain percentage of animals. The specificity of the reaction is indicated by the fact that there were no deaths among animals pretreated with pertussis vaccine alone and challenged with bovine serum, or among animals that had been sensitized with the mixture of bovine serum and pertussis vaccine, but were challenged with rabbit serum or egg albumin.

Effect of allergic shock on fate of staphylococci in the organs of mice. It has been observed in preliminary tests, not reported here, that the susceptibility to tuberculosis of mice sensitized to bovine or rabbit serum can be increased by injecting the tubercle bacilli in association with small amounts of the type of serum used for sensitization. As pertussis vaccine increases the ability of mice to develop the allergic state, an experiment was carried out to determine whether the concentration of this adjuvant used at the time of sensitization with bovine serum would influence the response of animals infected with bacteria resuspended in serum.

Four groups of female mice, 5 weeks old, of the so-called Rockefeller Institute Swiss breed, were pretreated by intraperitoneal injection of 0.5 ml bovine serum, with or without admixture of different amounts of pertussis vaccine (Sharp and Dohme). The preparation of pertussis vaccine used in this experiment was an experimental batch (not packaged for human use) which had been kindly supplied by Dr. J. Munoz of Merck, Sharp and Dohme. It must be noted that this preparation of vaccine was much more concentrated (approximately 15 times) than

TABLE II. Specific Sensitization of Mice* to Bovine Serum.

Sensitizing treatment (i.p.)		Challenge inj. (i.v.)		
Serum (ml)	Pertussis vaccine (ml)	Time after sensitization (days)	Material inj. (ml)	Deaths† (out of 5)
.5	.05	14	.2 bovine ser.	5
			.1	5
			.05	3
			.02	4
			.005	4
			.001	0
0			.2	0
0			.1	0
.5	.05	19	.01 *Idem*	5
			.001	3
			.001	1
			.0005	0
			.2 rabbit ser.	0
			.01 " "	0
			.2 1% egg alb.	0
			.05 " "	0

* Female CF_1 mice, 7 wk old.
† All deaths occurred within 30-60 min.

TABLE III. Effect of Amount of Pertussis Vaccine Used as Adjuvant on Susceptibility of Mice to Staphylococcal Infection.

Sensitizing treatment,* pertussis vaccine (ml i.p.)	Challenge infection† (i.v.): 0.1 ml staph. culture + 0.1 ml 1/1000 bovine serum										
	Deaths within 4 hr	No.‡ of staph. colonies recovered from individual organs									
		Kidneys					Lungs				
.02	3/5	2	12				4	10			
.006	0/5	1	70	400	1100	3000	10	10	10	300	300
.0006	1/5	5	100	1700	5000		100	240	250	3000	
0	0/5	11	17	100	300	1200	30	30	30	290	500

* All animals received 0.5 ml bovine serum i.p.

† 27 days after sensitization.

‡ Figures to be multiplied by 1,000 for whole lung, and by 10,000 for whole kidney.

the one from Lederle Laboratories used in the 2 preceding experiments. Twenty-seven days later, all animals received a mixture containing 0.1 ml of culture of *Staphylococcus aureus* (Smith strain) and 0.1 ml of 1/1000 bovine serum in saline. The characteristics and behavior of this staphylococcal strain, *in vitro* and *in vivo*, have been reported earlier(4). The animals were sacrificed 24 hours after infection and the numbers of staphylococcal colonies that could be recovered from their organs were determined by technics which have been described elsewhere(5). The results are presented in Table III.

Three of the animals which had received the largest amount of pertussis vaccine along with the sensitizing dose of bovine serum died acutely upon receiving the 1/2000 serum dilution used as diluent for the infective dose 27 days after sensitization. It is probable that this finding can be explained by the fact that the degree of allergic sensitization to bovine serum was the highest in animals that had received the largest amount of vaccine.

There was no evidence that the allergic shock had affected the response to infection, as no striking differences could be detected between the numbers of staphylococcal colonies recovered from the organs of animals sensitized with bovine serum alone and from animals having received in addition .006 or .0006 ml pertussis vaccine. While this experiment was in progress, however, the results of other unrelated studies disclosed that vaccination of mice with pertussis vaccine had a marked and lasting protective effect against infection with staphylococci. Evidence of this protective effect has been presented in a recent publication(6). Suffice it to state here that mice vaccinated with an amount of pertussis vaccine as small as 0.0006 ml (Sharp and Dohme preparation) still exhibit several weeks later a much increased resistance to staphylococcal infection. In view of this fact, it became evident that the protective effect of pertussis vaccine alone would have to be controlled in any study of the influence exerted by allergic shock on susceptibility to infection of animals sensitized with the help of this material as adjuvant. The experiment

TABLE IV. Effect of Allergic Shock on Fate of Staphylococci in the Organs of Mice.

Sensitizing treatment,* bovine serum (ml i.p.)	Time (days) between sensitizing and challenge	Challenge infection (i.v.): 0.1 ml staph. culture + 0.1 ml 1/1000 bovine serum											
		Nos. of staph. colonies† recovered from individual organs											
		Kidney				Liver				Lungs			
.5	10	4	14	85		27	39	103		13	22	31	
0	"	.2	1	12		53	140	148		.2	2	?	
.5	18	8	55	160	1900	24	210	310	1100	3	6	17	210
0	"	.1	1	5	35	5	48	56	230	0	0	.4	2
.5	23	560	3200	3500		78	170	300		0	1	4	
0	"	25	80	750		12	17	47		.1	1	1	

* All animals received .05 ml pertussis vaccine (Lederle) i.p.

† To be multiplied by 10^3 for whole lungs, and by 10^4 for whole liver and kidney.

presented in Table IV was planned with this requirement in mind.

It is seen that, whatever the time of infection, more colonies were recovered from the kidneys, livers, and lungs of animals that had been pretreated with bovine serum and with pertussis vaccine than from the organs of animals that had received the vaccine alone.

These findings suggest that the severity of the infectious process had been increased, at least temporarily, by the mild allergic shock resulting from the presence of 1/2000 bovine serum in the diluent used for injecting the bacterial suspension.

Summary. 1) Female albino mice of two different breeds were treated intraperitoneally with a mixture of bovine serum and pertussis vaccine. In confirmation of findings of other investigators, it was found that a large percentage of the animals so sensitized died within 30-60 minutes following the intravenous injection of very small amounts of serum 2-4 weeks after sensitization. The sensitizing effect appeared specific as no deaths occurred when either rabbit serum or egg albumin was used for the challenge injection. 2) Mice pretreated by intraperitoneal injection of bovine serum and pertussis vaccine, or of pertussis vaccine alone, were infected intravenously several weeks later with staphylococci resuspended in 1/2000 bovine serum. When they were sacrificed 24 hours after infection, it was found that many more staphylococcal colonies could be recovered from the kidneys, liver and lungs of the animals that had been sensitized to bovine serum, than from the organs of animals of the control group.

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1. Parfentjev, I. A., and Goodline, M. A., *J. Pharmacol. and Exp. Therap.*, 1948, v92, 411.

2. Malkiel, S., and Hargis, B. J., Proc. Soc. Exp. Biol. and Med., 1952, v81, 109.

3. Malkiel, S., Hargis, B. J., and Feinberg, S. F., *J. Immunol.*, 1953, v71, 311.

4. Smith, J. Maclean, and Dubos, R. J., *J. Exp. Med.*, 1956, v103, 87.

5. Pierce, C. H., Dubos, R. J., and Schaefer, W. H., *ibid.*, 1953, v97, 189.

6. Dubos, R. J., and Schaedler, R. W., *ibid.*, 1956, v104, 53.

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Effect of Aspirin and Reserpine on Adrenocortical Response to Piromen in Man. (22751)

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The elevated body temperature resulting from parenterally administered pyrogen in man is associated with a rise in the urinary output of 17-ketosteroids, corticosteroids, and an increase in concentration of free 17-hydroxycorticosteroids (17-OHCS) in plasma (1-3). Administration of aminopyrine has been shown to block the febrile response to typhoid-paratyphoid vaccine without altering the associated hemodynamic changes(4,5). Christy *et al.* have shown that aminopyrine also blocks the adrenocortical response to typhoid vaccine(6). Intravenously administered reserpine in dosages up to 1 mg/kg body weight failed to block the temperature elevation resulting from the injection of colon bacilli in rabbits(7). More recently Windle has noted a blocking effect of reserpine on fever production and eosinopenia following Piromen administration in monkeys (personal communication). Other studies in mice have reported that reserpine causes hypothermia (8).

The present study has been designed to elucidate the nature of the adrenocortical response to administration of a bacterial pyrogen, Piromen, and to observe the effects of aspirin and reserpine when given in conjunction with Piromen. Specifically, test situations are aimed at clarifying the following points: 1. Is the increase in plasma free 17-OHCS concentration observed following Piromen due to increased adrenocortical secretion or decreased removal from the miscible pool? 2. Is hydrocortisone the adrenocortical secretion responsible for elevated plasma 17-OHCS levels following Piromen administration? 3. What is the effect of aspirin on the adrenocortical response to Piromen? 4. What is the effect of reserpine on the adrenocortical response to Piromen? 5. Is fever the cause of elevated 17-OHCS concentration resulting from the administration of Piromen?

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Methods. Plasma free 17-OHCS were determined by the method of Silber and Porter (9), as modified by Peterson *et al.*(10). Hydrocortisone and corticosterone were determined specifically by isotope dilution(11,12). Eosinophil counts were made in a Speirs-Levy eosinophil counting slide. A. *Determination of Half-Life of Hydrocortisone.* Plasma half-life of hydrocortisone was determined in 4 young, normal subjects following intravenous administration of 0.3-0.4 μg Piromen/kg/body weight on one day and Piromen placebo on the other day. Three subjects received intravenous injections of 200 mg hydrocortisone sodium succinate and the 4th subject received 20-minute infusions of 200 mg free hydrocortisone in ethanol-water solution. Blood samples were withdrawn before administration of Piromen or Piromen placebo, immediately prior to hydrocortisone administration, and at appropriate intervals thereafter. Plasma 17-OHCS concentrations were determined for all specimens. B. *Determination of Miscible Pool and Turnover Rate of Hydrocortisone.* The miscible pool and turnover rate of hydrocortisone were determined on 1 normal, male subject, age 19, by the method of Peterson *et al.*(13). The study was carried out on each of 2 different days; 1 day the subject received 0.2 μg Piromen/kg body weight 2 hours prior to injection of hydrocortisone-4-C^{14}; on another day, he received Piromen placebo 2 hours before injection of hydrocortisone-4-C^{14}. C. *Effect of Aspirin and Reserpine on Plasma 17-OHCS Levels.* A total of 8 normal subjects, 4 males and 4 females, between 18 and 21 years of age, received in randomized order, saline infusion, Piromen, and Piromen plus aspirin. Observations made on days of saline infusions were used as control data. (The saline infusions do not alter normal diurnal fall in plasma 17-OHCS.) Three of the above subjects also received Piromen plus reserpine. Saline infusions were given from 8 to 10 A.M. at 3 ml/min. Piromen was administered intravenously (0.1 μg/kg body weight) at the beginning of each test (8 A.M.). Days on which the effect of Piromen, or Piromen plus reserpine, was determined, the subject was given 2 lactose capsules (identical in appearance to aspirin capsules) at 7 A.M., 9 A.M., 12 M., and 3 P.M. Days on which Piromen plus aspirin was tested, 2 capsules each containing 0.33 g of aspirin were given at same times. Subjects participating in the Piromen plus reserpine study were primed with oral reserpine in the following manner: first day, 0.25 mg reserpine q.i.d.; second day, 0.5 mg reserpine q.i.d.; third day, 0.75 mg reserpine q.i.d.; fourth and fifth days, 1.0 mg reserpine q.i.d. The test was carried out on the fifth day. The first blood sample was drawn immediately before administration of the test solution (8 A.M.). Blood was then withdrawn at hourly intervals until 12 M. and then at 2 P.M. and 4 P.M. Venous blood was obtained at 8 A.M. and 12 M. for eosinophil counts. Pulse and auscultatory blood pressure recordings were made hourly from 8 A.M. through 4 P.M. Oral temperatures were determined at 8 A.M., 10 A.M., 12 M., and 3 P.M. Fever is defined as an oral temperature above 37°C.

Results. Nature of Adrenocortical Secre-

TABLE I. Effect of Piromen on Concentration of Phenylhydrazine-Reacting Substances, Hydrocortisone and Corticosterone in Plasma.

		Phenylhydrazine method	Isotope dilution method
		μg/100 ml plasma	
Hydrocortisone	Control	15	15
	Piromen	24	25
Corticosterone	Control	—	1.8
	Piromen	—	3.0

TABLE II. Effect of Piromen on Hydrocortisone Half-Life.

Subject	Hydrocortisone preparation	Half-life (min.)	
		Placebo	Piromen
DH	Sodium succinate	78	70
WG	*Idem*	113	110
WM	"	95	64
LS	Free	132	114
Avg		104	90

tion Following Piromen Administration. A comparison of the plasma concentration of 17-OHCS as determined by the phenylhydrazine method with the plasma concentration of hydrocortisone determined by the specific isotope dilution method shows that the concentration of 17-OHCS in the plasma during the control and the Piromen test is equal to the hydrocortisone concentration (Table I). Therefore, the Silber-Porter chromogens will be designated as hydrocortisone. By use of the isotope dilution procedure, it has also been shown that Piromen causes an increase in plasma corticosterone (Table I).

Miscible Pool and Turnover Rate of Hydrocortisone After Piromen. To ascertain whether the increase in plasma hydrocortisone is due primarily to decreased removal from the miscible pool or increased adrenocortical secretion, 2 tests were employed. First, the plasma half-life of hydrocortisone was determined approximately 3 hours following injection of Piromen or Piromen placebo. Table II indicates that the plasma half-life of hydrocortisone is not prolonged by prior administration of Piromen but rather is decreased by an average of 13%. Second, to determine more definitively if the increased plasma hydrocortisone concentration following Piromen administration was due to an increased secretion of hydrocortisone, the miscible pool and turnover rate of hydrocortisone were determined using hydrocortisone-4-C^{14}. Table III presents the data in 1 normal subject on a control day and on a day he received Piromen. From this Table, it can be seen that the miscible pool was increased 5-fold and the rate of secretion of hydrocortisone was increased 6-fold.

TABLE III. Effect of Piromen on Miscible Pool and Turnover Rate of Hydrocortisone.

	µg % plasma F	Miscible pool, mg	Turnover rate, mg/hr
Control	14	1.5	.75
Piromen	35	7.6	4.70

Influence of Aspirin on Elevated Plasma Hydrocortisone Levels Observed With Piromen. Fig. 1 shows the mean hydrocortisone levels for 8 matched pairs receiving: 1) saline control infusion; 2) Piromen, and 3) Piromen plus aspirin. Intravenous injection of 0.1 µg Piromen/kg causes significant[†] increases in plasma hydrocortisone concentration for 8 hours following its administration. Hydrocortisone levels resulting from concomitant administration of Piromen and aspirin are significantly lower than those observed with Piromen alone at the 2nd and 3rd hours following the injection of Piromen. However, this dosage of aspirin is not sufficient to counteract completely the Piromen effect since there is a significant elevation of plasma hydrocortisone over saline control values at the 3rd through 6th hour of the test and probably a significant elevation at the 2nd and 8th hours ($P < 0.05$).

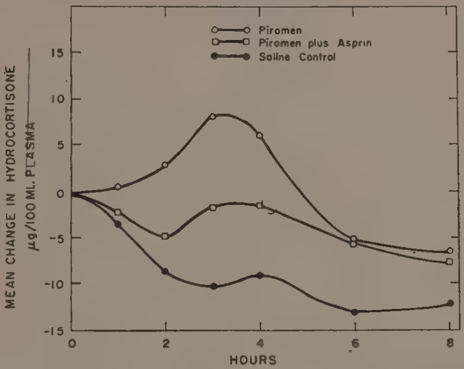

FIG. 1. Effect of Piromen, Piromen plus aspirin, and saline on plasma hydrocortisone levels. Individual points represent mean values for group of 8 normal subjects.

Influence of Reserpine on Elevated Plasma Hydrocortisone Levels Observed With Piro-

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[†] The term "significant" is used to indicate statistical significance at the 1% level of confidence as determined by the t-test of Fisher.

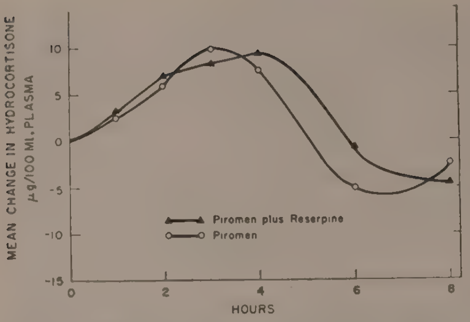

FIG. 2. Effect of Piromen and Piromen plus reserpine on plasma hydrocortisone levels. Individual points represent mean values for group of 3 normal subjects.

men. Fig. 2 shows the mean plasma hydrocortisone levels associated with Piromen and Piromen plus reserpine for 3 subjects. It is apparent that reserpine administered in this manner does not depress adrenocortical response to Piromen.

Observations on Temperature, Eosinophil Counts, Pulse, and Blood Pressure. Fig. 3 shows the temperature course during the different test procedures. Compared with the control observations, Piromen causes a significant elevation in temperature at 12 M. Aspirin administration causes a significant decrease in the temperature response to Piromen at 12 M. and 3 P.M. The 8 A.M. temperatures observed on 5 subjects reserpinized in the manner described (the 3 subjects included in the adrenocortical evaluation and 2 additional) were not lower than the 8 A.M. temperatures on these same individuals on control and Piromen test days. Reserpine exerts no blocking effect on the temperature response to Piromen. The average percentage fall in the circulating eosinophils 4 hours after the beginning of the test was: saline, 39%; Piromen plus aspirin, 39%; Piromen, 58%; and Piromen plus reserpine, 57%. The 4-hour fall in the number of circulating eosinophils following Piromen is significantly greater than that following saline or Piromen plus aspirin. This dosage of Piromen causes a significant tachycardia from the 3rd through 7th hour of the test when compared with control values. Aspirin administration with the Piromen significantly lowers the pulse rates from the 4th through 8th hour. Reserpine causes bradycardia but the percentage increase in pulse rate following Piromen administration is not altered. No differences in the base-line blood pressure or blood pressure responses following Piromen or saline were noted between any experimental groups.

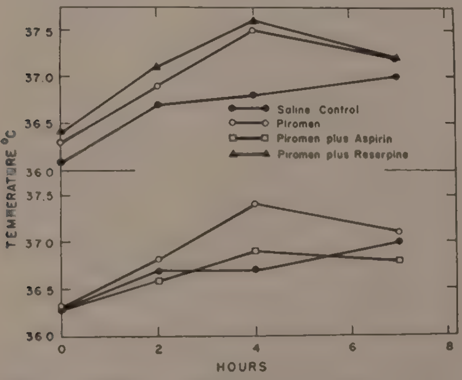

FIG. 3. Effect of saline, Piromen, Piromen plus aspirin, and Piromen plus reserpine on temperature. Upper graph represents mean values of 3 subjects. Lower graph represents mean values of 8 subjects.

Relationship Between Body Temperature and Plasma Hydrocortisone Concentration. Analysis of individual relationship between plasma hydrocortisone concentrations and temperature rise 2 hours after Piromen administration indicates that only 1 of the 8 subjects revealed a temperature rise greater than 37°C. There is no significant difference in the mean 10 A.M. temperature between Piromen and control groups, while the plasma hydrocortisone levels are significantly higher with Piromen administration (mean difference is 12.9 µg%). Two hours after combined Piromen and aspirin administration, 2 of the 8 subjects showed a rise in plasma hydrocortisone (albeit less than they showed with Piromen alone) with a temperature less than 37°C. A 3rd subject manifested a temperature greater than 37°C, but there was no associated rise in the plasma hydrocortisone concentration.

Two hours after Piromen administration, 2 of the 3 subjects receiving reserpine manifested temperatures above 37°C, but all 3 subjects showed a rise in plasma hydrocortisone levels.

Comments. One of the observations of major interest in this study was the appearance of significantly increased adrenocortical secretory activity distinctly before the appearance of fever. Indeed, only one of the subjects receiving Piromen manifested an oral temperature above 37.6°C at the height of the temperature response. It is apparent that the effect of Piromen on adrenocortical activity is not dependent on fever production. It appears that the elevation of temperature and of plasma hydrocortisone levels are separate manifestations of the effect of Piromen. The inference of Christy *et al.*(6) that fever is the cause of increased plasma concentrations of 17-OHCS secondary to pyrogen administration is not supported by our observations.

The likelihood of salicylates in the dosages employed in this study causing a significant alteration in plasma 17-OHCS levels may be discounted on the basis of studies of Done *et al.*(14). These investigators were unable to show a significant rise or fall in plasma 17-OHCS levels associated with the administration of 1 gram of sodium salicylate administered hourly for 8 hours. Furthermore, Peterson and Bunim (unpublished observations) have shown that salicylates in a dose of 8 g/24 hrs exert no effect on the turnover rate of hydrocortisone in human subjects.

The difference in effect of reserpine on pyrogenic and eosinopenic effect of Piromen in monkeys observed by Windle (personal communication) and in our human subjects may be explainable by the dosages used. Windle employed a dose of 20 mg reserpine/kg body weight and a dose of 0.5-1 μg Piromen/kg body weight.

The studies on the plasma half-life of hydrocortisone and the miscible pool and turnover rate of hydrocortisone-4-C^{14} discount the possibility that factors other than increased adrenocortical secretion are responsible for the increased plasma 17-OHCS levels resulting from Piromen administration. It is apparent that the increased plasma hydrocortisone half-life which follows Piromen administration to anesthetized dogs(15) does not occur in unanesthetized humans.

Conclusions. 1. Intravenous administration of 0.1 μg Piromen/kg body weight to young, normal male and female subjects causes a significant rise in body temperature and plasma hydrocortisone levels. 2. Increased concentration of plasma free 17-OHCS following Piromen administration has been shown to be the result of increased secretion of hydrocortisone. 3. The rise in plasma hydrocortisone distinctly precedes the elevation of body temperature above 37°C. Fever is, therefore, not the cause of the elevated plasma hydrocortisone levels. 4. The increases in plasma hydrocortisone levels, eosinopenia, temperature, and pulse rate caused by Piromen administration are significantly lessened by the concomitant administration of aspirin. 5. Administration of reserpine in a dose of 4 mg/24-hr period caused no hypothermia in normal human subjects. 6. Pyrogenic, eosinopenic, and adrenocortical stimulatory effects of Piromen are not altered by the prior and concomitant administration of reserpine.

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1. Rosen, D. A., *Am. J. Ophthalmol.*, 1953, v35, 1783.

2. Conn, J. W., Fajans, S. S., Louis, L. H., Seltzer, H. S., and Kaine, H. D., *Recent Prog. Hormone Res.*, 1954, v10, 471.

3. Bliss, E. L., Migeon, C. J., Eik-nes, K., Sandberg, A. A., and Samuels, L. T., *Metabolism*, 1954, v3, 493.

4. Bradley, S. E., Chasis, H., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1945, v24, 749.

5. McDonald, R. K., Solomon, D. H., and Shock, N. W., *ibid.*, 1951, v30, 457.

6. Christy, N. P., Donn, A., Jailer, J. W., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 453.

7. Bein, H. J., *Experientia*, 1953, v9, 107.

8. Deming, I., Bogdanski, D. F., Udenfriend, S., Shore, P. A, Brodie, B. B., *Fed. Proc.*, 1956, v15, 416.

9. Silber, R. H., and Porter, C. C., *J. Biol. Chem.*, 1954, v210, 923.

10. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., Bunim, J. J., *J. Clin. Invest.*, 1955, v34, 1779.

11. Peterson, R. E., Karrer, A., and Guerra, S. L.,

Anal. Chem.

12. Peterson, R. E., *J. Biol. Chem.*

13. Peterson, R. E., and Wyngaarden, J. B., *J. Clin. Invest.*, 1956, v35, 552.

14. Done, A. K., Ely, R. S., and Kelley, V. C., *Metabolism*, 1955, v4, 129.

15. Eik-nes, K. B., *Fed. Proc.*, 1956, v15, 247.

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Effect of Synthetic Lysine-Vasopressin on Plasma Hydrocortisone Levels in Man. (22752)

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Previous studies in this laboratory(1) have demonstrated that intravenous administration of highly purified arginine-vasopressin and highly purified lysine-vasopressin, as well as Pitressin, cause increased plasma hydrocortisone concentrations in man. Because of the possibility of ACTH-releasing or adrenotrophic substances contaminating these vasopressin preparations, a comparative study has been made of the effects of synthetic lysine-vasopressin and Pitressin on plasma hydrocortisone levels.

Methods. Five normal subjects, aged 18 to 21 years, were assessed for plasma hydrocortisone responses to intravenously administered Pitressin and synthetic lysine-vasopressin[†] in the following manner: At 8 a. m. on 2 different test days, each subject received intravenously 2 ml of vasopressin diluent (0.5% chlorobutanol solution adjusted to pH 3.6 with acetic acid). At 8:30 a. m., the subjects received intravenously on 1 test day 2 U. Pitressin and on the other test day, 2 U. of synthetic lysine-vasopressin. The order in which each subject received the 2 vasopressin preparations was randomized. Venous blood samples of 20 ml each were obtained through an indwelling Cournand needle prior to administration of the diluent (8 a. m.), 30 minutes following injection of diluent (*i.e.*, immediately preceding injection of the vasopressin preparation), and 30 minutes following the injection of vasopressin (9 a. m.). Plasma hydrocortisone concentrations were determined in duplicate on all samples by the method of Silber and Porter(2), as modified by Peterson *et al.*(3). Pulse rates and auscultatory blood pressures were determined at 1-minute intervals before, during, and after injection of Pitressin and synthetic lysine-vasopressin.

TABLE I. Effect of Pitressin and Synthetic Lysine-Vasopressin on Plasma Hydrocortisone Concentrations.

	Plasma hydrocortisone, μg/100 ml plasma	
Time of blood withdrawal	2 U. Pitressin	2 U. synthetic lysine-vasopressin
0′ (Pretest value)	16.6	18.0
30′ Following inj. of vasopressin diluent	14.3	16.6
30′ Following inj. of vasopressin preparation	25.7	25.6

Results. Table I shows the mean plasma hydrocortisone[‡] concentrations for the 5 subjects on the Pitressin test day and on the synthetic lysine-vasopressin test day. There is no significant difference between the plasma levels elicited by 2 U. of Pitressin and 2 U. of synthetic lysine-vasopressin. The mean blood pressure response to both vasopressin preparations was the same (approx. 20 mm Hg increase) while the pulse rates were un-

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[*] NIH, Public Health Service, U. S. Dept. of Health, Education, and Welfare.

[†] We wish to express our appreciation to Dr. Vincent du Vigneaud, Cornell University Medical College, for supplying us with synthetic lysine-vasopressin.

[‡] It has been shown in other studies(4,5) that the Silber-Porter chromogens are equivalent in concentration to free hydrocortisone.

affected by either.

Summary. 1. In man, the intravenous injection of synthetic lysine-vasopressin causes a rise in plasma hydrocortisone concentration comparable to that following the intravenous administration of Pitressin. The effect of the latter is therefore not due to impurities. 2. The pressor effect of synthetic vasopressin is of the same order as that noted following the injection of Pitressin.

1. McDonald, R. K., and Weise, V. K., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 481.

2. Silber, R. H., and Porter, C. C., *J. Biol. Chem.*, 1954, v210, 923.

3. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bunim, J. J., *J. Clin. Inv.*, 1955, v34, 1779.

4. Peterson, R. E., Karrer, A., and Guerra, S. L., *Anal. Chem.*

5. McDonald, R. K., Weise, V. K., and Peterson, R. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v93, 343.

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Some Effects of Parathyroid Extract and Cortisone on Metabolism of Strontium and Calcium.* (22753)

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Diuresis with an increased urinary excretion of calcium and phosphorus results when parathyroid extract is administered intraperitoneally to rats(1,2). After injection of the hormone into normal animals the blood calcium rises followed by an accompanying rise in urinary calcium. Burrows(3) reported that the rise of blood calcium following injection of parathyroid extract is a result of mobilization of calcium from bones. The subepiphyseal plate appears to be the region of the bone from which the parathyroid extract brings about removal of calcium(4). The kidneys of rats receiving parathyroid extract contained 365 times more calcium than the controls when observed within 24 hours after receiving the extract containing the hormone. However, after the first 24 hours no significant difference was observed between treatments. Cortisone has been found to increase excretion of potassium, sodium and chloride in normal dogs. Its relation to calcium deposition in soft tissues and bone growth(5,6) has been reported from several laboratories.

The investigation here reported was undertaken to determine whether strontium would respond similarly to calcium when experimental animals receive parathyroid extract and also whether cortisone would counteract deposition of these minerals in kidney tissue in rats receiving parathyroid extract.

Methods. Two experiments were conducted during these studies. Uniform Rockland rats weighing approximately 160 g were used for these experiments. The rats were housed in metabolism cages for ease of making balance studies as outlined by Hansard and Comar (7). In each experiment the rats were given 5 μc of calcium-45 or strontium-89, subcutaneously 24 hours before receiving injections of the hormones. The parathyroid extract was administered subcutaneously in doses of 100 I. U. each, 24, 32, 48 and 56 hours after receiving the nuclide. When cortisone was administered, it was given in 4 subcutaneous injections of 25 mg in a saline solution, at the same time as the parathyroid extract. Sixteen hours after receiving the final hormone

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TABLE I. Relation of Parathyroid Extract to Calcium and Strontium Metabolism.* Six rats per series.

Treatment	Nuclide	Femur, %/femur	Femur, %/mg Ca.	Urinary excretion, %/rat 24 hr	48 hr	72 hr	Kidney, %/g (wet wt)	Fecal excretion, %/rat 24 hr	48 hr	72 hr
None	Sr-89	4.2	.035	5.3	1.36	.43	.02	5.9	4.3	1.24
PTH	"	4.7	.041	5.4	1.11	.74	.26	6.5	5.0	.94
None	Ca-45	4.7	.034	1.7	.24	.13	.00	5.3	2.3	.99
PTH	"	3.6	.022	2.9	.30	.59	.34	5.2	2.5	.78

* Expressed as % of administered dose.

injection the rats were sacrificed and the femur and kidneys assayed for calcium-45 or strontium-89. The daily urine and feces were collected and assayed for the radionuclide being studied. All samples were dry-ashed at 650°C, dissolved in concentrated hydrochloric acid and an aliquot dried in a stainless steel counting cup and counted under a Geiger-Muller tube. Total calcium was determined by precipitating calcium as oxalate and titrating the precipitate with potassium permanganate using the method described by Comar *et al.*(8).

Results. Since the rat uses calcium in preference to strontium, the first experiment was designed to study the influence of parathyroid extract on both calcium and strontium metabolism and this relationship. The results are summarized in Table I. Parathyroid extract influenced the metabolism of strontium and calcium similarly as related to deposition in kidney tissue and urinary excretion. Statistical analysis of the data indicated a significant difference at the 5% level of confidence between treatments in 72 hour urinary excretion and kidney tissue deposition. Parathyroid extract caused diuresis, increased urinary excretion of calcium and strontium during 24 to 48-hour period after administration of the hormone and increased deposition of these nuclides in the kidneys. The amount removed from the bones was not of sufficient magnitude to detect differences by analysis of bones or autoradiographs.

In the second experiment the relation of cortisone to the influence exerted by parathyroid extract on calcium and strontium metabolism was studied. Parathyroid extract, alone or with cortisone, significantly (5% level) increased urinary excretion of calcium and strontium during the 24 to 48-hour period after administration of the hormone over the control rats. The significant increase in calcium and strontium deposition in the kidney tissues observed when parathyroid extract was administered did not appear when cortisone was administered with parathyroid extract. Cortisone prevented the accumulation of both calcium and strontium in kidney tissue. It is interesting to observe that whereas parathyroid extract increases both urinary and kidney calcium and strontium: cortisone influences

TABLE II. Relation of Parathyroid Extract and Cortisone to Calcium and Strontium Metabolism.*

Treatment	No. rats	Nuclide	Femur, %/femur	Urinary excretion, %/rat 24 hr	48 hr	72 hr	Kidney, %/g (wet wt)	Fecal excretion, %/rat 24 hr	48 hr	72 hr
None	4	Sr-89	3.2	6.2	1.13	.61	.014	8.5	4.12	2.28
PTH	6	"	3.1	6.9	1.61	1.45	.390	8.4	4.23	1.07
PTH + cortisone	6	"	2.9	8.8	1.48	1.50	.014	8.6	5.09	1.06
None	4	Ca-45	1.9	1.6	.21	.09	.037	1.8	.71	.44
PTH	6	"	2.1	1.5	.19	.22	.166	2.0	.68	.53
PTH + cortisone	6	"	2.1	2.1	.36	.27	.032	2.2	.97	.43

* Expressed as % of administered dose.

only the accumulation in the kidney tissue. Cortisone did not influence the diuresis caused by the parathyroid extract.

Summary. 1) Parathyroid extract increased the urinary excretion of calcium and strontium as well as increased deposition of these minerals in the kidney tissue. 2) Cortisone prevents accumulation of strontium and calcium in kidney tissue but does not influence urinary excretion or diuresis.

1. Ellsworth, R., and Nicholson, W. H., *J. Clin. Invest.*, 1935, v14, 823.

2. Talmage, R. V., and Kraintz, F. W., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 263.

3. Burrows, R. B., *Am. J. Anat.*, 1938, v62, 237.

4. Talmage, R. V., Lotz, W. E., and Comar, C. L., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 578.

5. Follis, R. H. F., *ibid.*, 1951, v76, 722.

6. Sissons, H. A., and Hadfield, G. J., *J. Anat.*, 1955, v89, 69.

7. Hansard, S. L., and Comar, C. L., *Nucleonics*, 1953, v7, 44.

8. Comar, C. L., Hansard, S. L., Hood, S. L., Plumlee, M. P., and Barrentine, B. F., *Nucleonics*, 1951, v8, 19.

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Deoxyribonucleic Acid Content and Mitotic Activity of Vagino-Cervical Epithelium in Colchicine-Treated Mice During Estrus.* (22754)

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The estrous cycle of the mouse is characterized by alternating periods of rapid cell growth and involution of genital tissues and affords an excellent opportunity for study of fundamental chemical aspects of cellular growth in response to hormonal stimulation. Quantitative cytochemical studies of estrus have been limited largely to the study of resting cells of uterine glands, to surface epithelial cells of rat uteri(1) and to total nucleic acid content of uteri. Greater accuracy of microspectrophotometric analysis of individual cells in elucidating their chemical status over chemical analysis of tissues containing heterogeneous cell types has been shown. The value of exfoliative cytology in following physiological and pathological events in the female genital tract has been well established(3-5). The present work was undertaken to study the deoxyribonucleic acid (DNA) content of exfoliated cervical and vaginal cells during the estrous cycle and to correlate these findings with DNA content and mitotic index of basal cells in tissue sections of vaginal fornix.

Materials and methods. Fourteen female C3H mice, 2 to 3 months of age and weighing 18 to 22 g, were divided into 6 groups. Groups 1 and 2 consisted of 3 animals each, Groups 3, 4, 5 and 6 of 2 animals each. Animals of Groups 5 and 6 were ovariectomized 10 days before start of experiment. Animals were kept in air-conditioned room (68-72° F), fed Purina Dog Checkers supplemented by daily addition of fresh bread and carrots. Daily vaginal smears were taken and animals sacrificed at various stages of estrous cycle. Animals of Group 6 were injected subcutaneously with 25 RU of theelin in peanut oil and sacrificed 10 and 20 hours later. Animals of Group 5 served as ovariectomized controls. Mitoses in basal cells of vagina and cervix were arrested in metaphase by the colchicine method of Bullough(6) in which period of treatment was limited to 5 hours to prevent interference with normal mitotic rate. Each animal received 0.1 mg of drug in 0.25 ml of normal saline subcutaneously and sacrificed by cervical dislocation 5 hours later. Smears were prepared immediately and stained by method of Papanicolaou(7). Vaginas and uteri were removed *in toto*, fixed in 10% buf-

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TABLE I. Nuclear DNA of 585 Nuclei and Mitotic Index of Basal Cells in Various Stages of Estrus following Colchicine Treatment, 5 Yr.

				Diploid cells			Tetraploid cells		
Group	No. of animals	Stage of estrus	Total nuclei counted	%	DNA, rel. units	% intermediate cells	%	DNA, rel. units	Mitotic index
1	3	Proestrus	150	82	1.03 ± .09	12.6	5.3	1.98 ± .21	40.2 ± .41
2	3	Estrus	85	67.3	1.07 ± .11	22.2	10.5	2.04 ± .30	223.8 ±10.2
			20*	100	1.01 ± .27	0	0		
			15†		.53 ± .31	0			
3	2	Metestrus	80	100	1.11 ± .22	0	0		9.5 ± 1.11
4	2	Diestrus	75	98.7	1.10 ± .16	1	0		7.3 ± .92
5	2	Castrates	75	100	1.08 ± .13	0	0		11.8 ± 1.23
6	1	Castrate + theelin, 10 hr	45	71	1.05 ± .17	22.2	6.7	2.10 ± .18	28.7 ± 3.2
	1	Castrate + theelin, 20 hr	50	64	1.06 ± .11	26	10	1.90 ± .22	48.3 ± 6.4

* Pyknotic nuclei. † Advanced pyknotic nuclei.

fered formalin, embedded in paraffin and sagittal serial sections cut at 7 and 15 μ thickness. 7 μ sections were stained with hematoxylin and eosin for mitotic counts. Sections cut at 15 μ were used for demonstration of DNA. They were subjected to the Feulgen reaction(8) carried out on smears and tissue sections after hydrolysis with N HCl at 60°C for exactly 12 minutes. Intact interphase basal, parabasal and keratinized cell nuclei selected at random in each vaginal smear stained by the Feulgen method were measured by a microspectrophotometer similar in design to that described by Pollister(9) and satisfying the requirements set by Pollister and Ornstein(10). A wavelength of 560 mμ was used, supplied by Bausch and Lomb grating monochromator. This wavelength represents maximum absorption peak of stain. To minimize light scattering, slides of smears were mounted in oil with a refractive index of 1.572, which matched that of unstained mucus. Relative amounts of DNA of individual nuclei in smears were obtained by multiplying extinction values by the nuclear area since all the nuclei were flattened(11). The major (a) and minor (b) axes of nuclei were measured with an optical micrometer and the area determined by the formula $A = \pi \frac{1}{2}a \times \frac{1}{2}b$. To include basal cells from both ectocervical and vaginal epithelium interphase basal cell nuclei were measured in cervical and vaginal portions of the vaginal fornix. Since care was taken to select uncut nuclei with no overlap, this sharply limited the number of nuclei measured, as cells were crowded especially during estrus. Relative amounts of nuclear DNA in tissue sections were obtained by the method of Swift(12).

Results. The results of DNA measurements in 550 basal and parabasal cell nuclei, 20 keratinized cells containing pyknotic nuclei, and 15 keratinized cells showing advanced pyknosis, karyolysis or karyorrhexis are presented in Table I. Representative histograms were prepared showing the DNA content of basal, parabasal and keratinized cells during various stages of estrus. Basal cells containing intermediate and tetraploid amounts of nuclear DNA were in vaginal smears and tissue sections during early proestrus, where keratinization was not of sufficient thickness to impair basal cell exfoliation. During estrus basal cells containing increased nuclear DNA, *i.e.* intermediates and tetraploids, were absent in vaginal smears while a considerable number were present in the tissues. Twenty hours following subcutaneous injection of theelin in ovariectomized mice the number of exfoliated basal cells containing increased nuclear DNA in smears had decreased, while basal cells containing increased nuclear DNA in the tissues had increased. Appearance of cells showing increased amounts of DNA paralleled the increased mitotic activity of vaginal and cervical basal cells during proestrus and estrus. During periods of quiescence (metestrus, diestrus and castrates), when mitotic activity was low, a constant diploid value of nuclear DNA was present (Table I). Even though some of the superficial cells exfoliated during estrus showed a marked degree of nuclear enlargement and early keratinization, the DNA content remained at a diploid value. A significant decrease in nuclear DNA occurred only in keratinized cells showing advanced pyknosis, karyolysis or karyorrhexis (Fig. 2).

Discussion. The stimulating effect of estrogenic hormones on growth of genital epithelium is well established, although the bulk of uterine growth following estrogen treatment is largely due to increase in cell volume rather than to cell multiplication(13). Allen *et al.*(14) utilizing colchicine to arrest mitoses have shown that estrogens actually markedly increase the rate of mitosis in vaginal and cervical epithelium of the mouse. The presence of numerous mitoses and interphase basal cells containing intermediate and tetraploid values of nuclear DNA in vaginal and cervical tissue during proestrus and estrus is in accord with this observation. This is in contrast to previous reports of constancy of nuclear DNA in hormonally stimulated uteri (1). However, several reasons may account for this difference. First, cells of vaginal and cervical epithelium where highest mitotic activity is seen, were not studied; secondly, due to the fact that vaginal and cervical epithelium represents but a small fraction of total uterine weights, a chemical determination of nucleic acids of the total uterus does not reflect the high degree of DNA synthesis occurring there. Bloch(15) found that although colchicine arrests cells in mitosis it does not interfere with DNA synthesis and that nuclei falling into higher DNA classes began to appear 10 hours after colchicine treatment. The period of colchicine treatment used in this study was of insufficient length to impair the rate of mitosis or to result in polyploidy due to DNA synthesis without subsequent mitotic division. The increase in nuclear DNA is probably due to a premitotic synthesis of DNA, as has been shown to occur in rapidly growing tissues by Patau and Swift(16). Furthermore, since Howard and Pelc(17) found that synthesis of DNA in the interphase nucleus occurs within a relatively short time is completed some time prior to mitosis, it is not surprising to find resting basal cells with increased amounts of nuclear DNA during periods of augmented growth. The preponderance of basal cells showing intermediate DNA values, and the relatively small tetraploid population, may indicate that once DNA synthesis has been completed division quickly ensues. This may explain the low mitotic index of uterine tissue following hormonal stimulation reported by Drasher and Salvatore(2,13). Basal cells containing increased amounts of DNA were present in vaginal smears taken during proestrus. The paucity of basal cells in smears during late proestrus and throughout estrus is due to the thick layer of keratin which prevents their exfoliation and is not a true reflection of the high degree of DNA synthesis present in the tissues during these periods.

Although cells exfoliated into the vaginal pool are more prone to cellular death and pyknosis, microspectrophotometric studies revealed that the majority of nuclei contained the normal diploid amount of DNA. This was true even though nuclear size was increased and early keratinized cells showing advanced pyknosis. This is in agreement with Leuchtenberger's report(18) on nuclear DNA changes during pyknosis.

It would appear from these experiments that the DNA content of exfoliated basal and parabasal cells in the vaginal pool is quite stable and within limits, corresponds to the DNA content of these cells in the cervical and vaginal mucosa during various physiological states. These findings indicate that except during high estrus it is possible to study DNA synthesis of epithelial cells during the estrous cycle by microspectrophotometric analysis of representative vaginal smears.

Summary. 1. Increased amounts of nuclear DNA were found in basal cells during periods of augmented growth in the estrous cycle. 2. These changes in nuclear DNA parallel the mitotic activity of the basal cells. 3. Exfoliated basal cells containing intermediate

and tetraploid amounts of DNA were found only during early proestrus. 4. The paucity of a tetraploid nuclear population during estrogenic stimulation may indicate rapid cellular division once DNA synthesis has occurred.

The author is indebted to Dr. Ruth Kleinfeld for helpful suggestions, and to Dr. Milton Lessler for use of laboratory facilities.

1. Gelfant, S., and Clemmons, J. L., *J. Cell. and Compl. Physiol.*, 1955, v46, 529.

2. Drasher, M. L., *J. Exp. Zool.*, 1952, v119, 333.

3. Stockard, C. R., and Papanicolaou, G. N., *Am. J. Anat.*, 1917, v22, 225.

4. Allen, E., *ibid.*, 1922, v30, 297.

5. von Haam, E., and Scarpelli, D. G., *Cancer Research*, 1955, v15, 449.

6. Bullough, W. S., *J. Exp. Biol.*, 1949, v26, 287.

7. Papanicolaou, G. N., *Science*, 1942, v95, 438.

8. DiStefano, H. S., *Chromosoma*, 1950, v3, 449.

9. Pollister, A. W., *Lab. Invest.*, 1952, v1, 106.

10. Pollister, A. W., and Ornstein, L., *Analytical Cytology*, edited by Mellors, R. C., Blakiston Co., New York, 1955, Chap. 1.

11. Swift, H. H., *The Nucleic Acids*, edited by Chargaff, E., and Davidson, J. N., Academic Press, New York, 1955, Chap. 17.

12. ———, *Physiol. Zool.*, 1950, v23, 169.

13. Salvatore, C. A., *Rev. Brasil. Biol.*, 1948, v8, 505.

14. Allen, E., Smith, G. M., and Gardner, W. U., *Am. J. Anat.*, 1937, v61, 321.

15. Bloch, D. P., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 341.

16. Patau, K., and Swift, H., *Chromosoma*, 1953, v6, 149.

17. Howard, A., and Pelc, S. R., *Exp. Cell Research*, 1951, v2, 178.

18. Leuchtenberger, C., *Chromosoma*, 1950, v3, 449.

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Bioenergetic Basis of Light-induced Fat Deposition in the White-crowned Sparrow.* (22755)

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It has been clearly established in at least 6 species of migratory birds that vernal premigratory deposition of fat in visceral and subcutaneous depots can be simulated by subjecting such birds to artificially prolonged daily photoperiods(1-6). That this phenomenon is the consequence of temporary hyperphagia and not the result of increased time for feeding, as daylength increases, appears evident from the experiments of Odum and Major(6) with white-throated sparrows (*Zonotrichia albicollis*) and Koch and de Bont(5) with a single chaffinch (*Fringilla coelebs*). These investigators, and Wallgren(7) also, have proposed that this response must involve the hypothalamus, a suggestion which is consistent with known effects of light on avian hypothalamo-hypophyseal system(8) and the role of hypothalamic subcenters in regulation of food intake in laboratory mammals(9-11). This investigation provides a more extensive and more quantitative basis for understanding this interesting phenomenon by *1.* definition of temporal course of fat deposition in wild populations of a typical migratory passerine bird (*Zonotrichia leucophrys gambelii*), *2.* measurement of energy intake during premigratory period in captive birds under natural conditions of light and temperature, and *3.* examination of bioenergetics of fat deposition obtained experimentally with prolonged daily photoperiods.

Material and methods. Animals: White-crowned sparrows (*Z. l. gambelii*) were captured from migrant and wintering populations in the vicinity of Pullman, Wash. Specimens for extraction of fat were weighed and killed immediately and kept frozen until extractions

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[†] National Science Foundation Predoctoral Fellow.

were made. Reserve stocks were maintained in large outdoor flight cages. These, as well as experimental birds, were fed a nutritionally adequate chick-starter mash having fat, protein, and carbohydrate components of 3.1%, 20.7% and 74.7% respectively, plus mineral and vitamin additives. The two batches had caloric contents of 4.439 and 4.552 kcal/g, respectively. *Fat determinations:* Entire carcasses were quantitatively extracted with diethyl ether in Soxhlet apparatus(15). Data are expressed as grams of ether-extractable lipid/100 g live body weight; this ratio is designated as *lipid index*. In all, 110 carcasses were extracted from collections in January to May, 1952, 1953, and 1954. The 1953 series of lipid indices, for the greater part, was obtained in conjunction with studies of morphology and composition of subcutaneous fat bodies(15,16). *Energy intake:* Birds were housed individually in Hendryx breeding cages (22 × 41 × 26 cm) from which floors had been removed. Each cage was placed in a close-fitting pan, 12.5 cm in depth, which permitted complete collection of excreta and spilled food. Food weighed to nearest 0.1 g was provided daily in hopper attached to side of cage; the unused feed from previous day was also weighed. Feed and water were continuously available. Moisture content of feed was determined weekly. Periodically (intervals of 4 to 7 days), pans were changed at a fixed hour. Spilled food and excreta were separated and dried to constant weight at 98° C. From these data the *gross energy* intake was calculated. Subtracting from this the total caloric value of excreta collected during the same period gives the *metabolizable energy*(14,17), which, except for energy potentially lost in specific dynamic action of food ingested and in bacterial fermentation, is the energy actually available to the bird. Calorimetric determinations of food and excreta were made with oxygen bomb calorimeter with experimental error of less than 0.5% between duplicate samples. Experimental error in de-

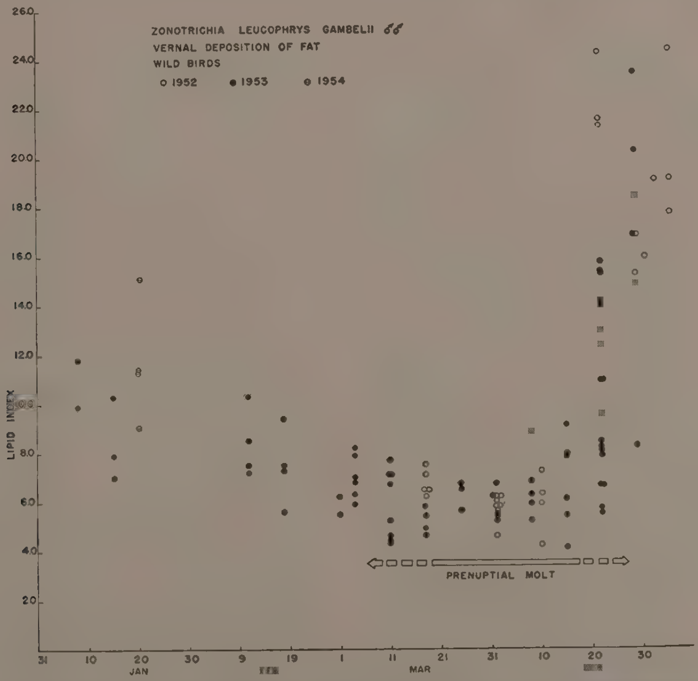

FIG. 1. Lipid indices in wild populations during late winter and spring. Migratory movement began during last 10 days of April.

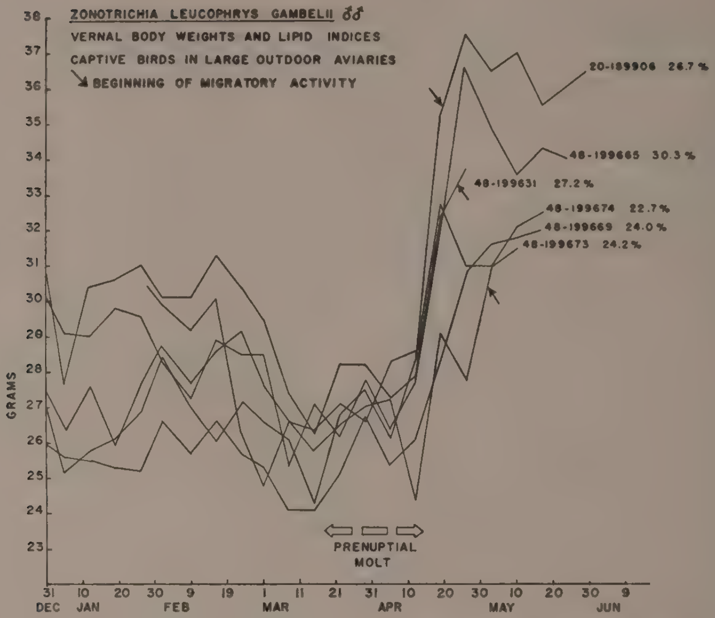

FIG. 2. Percentages at right are terminal lipid indices. Oblique arrows indicate onset of *Zugunruhe* in 3 birds in which activity was recorded.

termination of metabolizable energy is of the order of 3%. *Respiratory metabolism:* Oxygen consumption and carbon dioxide production were measured with apparatus similar to that of Schwabe and Griffeth(18). Attainment of *quasi*-basal conditions was guided by precautions suggested by Benedict(19) and Wallgren(7). *Activity recording:* Recording was by scheme similar to that described by Farner and Mewaldt(20) with the addition of impulse-counting circuits, which gave a numerical total for each hour for each cage. It must be emphasized that activity recording system of this type does not measure absolute or total activity but gives an *index of activity* as a fraction of total activity of a given subject. Since this fraction may differ among experimental animals, activity levels must be compared on a relative basis(21).

Results. Vernal Fat Deposition: Fig. 1 shows eruptive nature of the vernal premigratory fat deposition in nature. Migratory movement began during the last week of April in each of the 3 years. This pattern is similar to premigratory fat deposition for *Z. albicollis* in Georgia(22), except that the amount of fat deposited appears to be somewhat greater in *Z. l. gambelii.* This difference, however, might be only the result of differences in opportunity for collecting specimens during the first day or two of migratory movement. Odum and Connell(23) have recently shown that lipid indices of 25 to 30% are common in passerine birds captured in migration.

Fig. 2 demonstrates that the pattern of premigratory fat deposition, as indicated by a sample from the wild population, is substantially the same for individual birds held under natural conditions of temperature and photoperiod. Here it has been necessary to use body weight as an index of fat deposition. This is justifiable since our data and those of Odum and Perkinson(22) indicate an annual variation in fat-free weight of less than 10%. Therefore, variation in body weight of birds routinely weighed at fixed times of the day is due largely to variation in fat content. The terminal lipid indices in these captive birds are slightly higher than those of wild birds, probably because of somewhat restricted activity and readily available food supply. However, the date of beginning and rate of deposition of premigratory fat as well as temporal relationship with prenuptial molt are essentially the same. *Experiment 1:* The intake of metabolizable energy, the daily activity, and the variation in body weight were measured over 7-day intervals in 6 males confined in outdoor cages during the late winter and spring of 1955. It was found that a marked increase in body weight (fat deposition) in the last half of April was accompanied by a definite increase in energy intake. In 4 birds for which these data are acceptable, the increase was 30 to 50% above the preceding minimum. Although the energy *utilization coefficient* (metabolizable energy/gross energy × 100) increased in all birds from an average of approximately 70 in March to 76 in early June, the change was continuous and gradual and could not logically explain the very abrupt increase in metabolizable energy income. Likewise, ambient temperature oscillated only slightly around an increasing mean, with no extreme minima in April which can be correlated with timing or magnitude of maximum energy intake. Daylength, and consequently time for feeding, increased 9% (14.4 to 15.7 hours) during the last 3 weeks of April. A normal prenuptial molt occurred. The daily activity of birds continuously increased during the experiment, as previously shown(21) with *Z. l. gambelii* under the same conditions. Pronounced nocturnal unrest, or *Zugunruhe* (the behavioral homologue of migratory movement in caged migrants during migratory season)(24) developed on 28 April. *Exp. 2:* In mid-January, well after annual period of refractoriness to photic stimulation (25,26), 8 males were placed in individual metabolism-activity cages in constant-condition room maintained at 12 ± 2°C and 35-40% relative humidity. For 29 days these birds received 9 hours light per day (0900-1800) from incandescent lamps so arranged that light intensity measured at floor of cages was 35-40 foot-candles. A photoperiod of this duration is essentially subliminal for stimulation of testicular development and fat deposition. On Feb. 13 the group was divided into 2 groups of 4 each. One group was retained as untreated control. The experimental group was transferred to another room maintained at same temperature, humidity, and light intensity. The photoperiod for this group was then increased by 2-hour daily increments to 20 hours per day (lights out 1900-2300). The increase in photoperiod evoked patterns of hyperphagia and concomitant increase in body weight (fat deposition) which were very similar to those observed under natural conditions. A temporary increase in energy income of 27 to 43% above preceding minima occurred. The mean utilization coefficients in both groups were relatively constant at about 71 throughout experiment. The mean terminal lipid indices for experimental and control birds were 23.5 ($\sigma = 3.05$) and 12.3 ($\sigma = 3.34$) respectively. The difference is statistically significant ($P = 0.03$). Standard metabolic rates of controls and experimentals, computed on basis of lean body weight, show a sharp divergence after beginning of the increase in photoperiod, the experimentals uniformly attaining a rate approximately double that of controls. Respiratory quotients in the 2 groups ranged between 0.72 and 0.82. While it is very probable that this *apparent* increase in standard metabolic rate is due in part to a decrease in depth of sleep, as Bergman(29) has shown to occur in several passerine species during development of *Zugunruhe,* it is of particular importance here that there was no *decrease* in standard metabolic rate which could contribute energy to fat deposition. This aspect of our investigations will be considered in detail in subsequent publication. Activity of the 2 groups shows that there is no *decrease* in muscular activity of the 20-hour experimentals which could spare energy for fat deposition. By the end of the experiment all of experimentals were definitely in *Zugunruhe.* A very light molt occurred only in the spinal tract after the change to the 20-hour photoperiod. No molt was observed in the 9-hour controls.

Discussion. These observations suggest very strongly that the premigratory fat deposition in *Z. l. gambelii* is largely, if not entirely, the result of a photically induced hyperphagia. It is evident at least in the white-crowned sparrow, in the chaffinch(5), and the Ortolan bunting (*Emberiza hortulana*)(7) that premigratory adiposity cannot result from temporary decline in basal energy requirements as Groebbels(12) has suggested, nor from a combination of this and a moderate increase in food intake, as proposed by Merkel(13). Also it is apparent that there is no decrease in muscular activity nor increase in digestive efficiency of sufficient magnitude to account for the surplus of energy stored as fat. Our data apparently are inconsistent also with the hypothesis(14,24,27,28) that "productive" or surplus energy available for fat deposition is the result *primarily* of energy-sparing effects of increasing environmental temperature in spring, and the cessation of energy-demanding prenuptial molt plus increased energy intake made possible by more daylight for feeding. It seems unlikely that the eruptive nature and temporal precision of vernal fat deposition can be dependent to any substantial degree on such a fluctuating variable as environmental temperature, although it may have a secondary modifying role. With respect to the effect of increasing daylength, data from Exp. 1 show an average increase of 36% in rate of caloric intake (1.54 to 2.10 kcal/bird/hr of daylight) during a period when daylength increases by only 9%. Clearly there is no simple, linear relationship. The data also indicate that neither elevated environmental temperatures nor cessation of molt are essential in development of vernal adiposity. The simultaneous occurrence of molt and fat deposition has been noted previously under experimental conditions by Farner and Mewaldt(26) and Odum and Major(6).

In view of the similarity between development of obesity in mammals with hypothalamic lesions(11) and of premigratory fat deposition in birds it appears probable, as Koch and de Bont(5) have suggested, that this photically induced hyperphagia in birds is mediated by hypothalamic feeding centers. Although this hypothesis awaits the demonstration of feeding centers in the avian hypothalamus, such a mechanism controlled by vernal increase in daily photoperiod is consistent with observed annual precision of premigratory fattening.

Summary. In *Zonotrichia leucophrys gambelii* there is a striking deposition of fat in subcutaneous and visceral depots following prenuptial molt and preceding beginning of vernal migration. During the last 2 weeks of April, in natural populations, mean lipid indices increase from about 5% to more than 20%. A similar premigratory fat deposition occurs in captive birds under natural conditions of photoperiod and temperature. Similarly in these captive birds this fat deposition is followed by development of migratory behavior (*Zugunruhe*). Measurements of metabolizable energy intake in these birds show that fat deposition is accompanied by a marked increase in energy intake to levels of 30 to 50% above the preceding minima. This temporary hyperphagia represents an altered physiologic state rather than simply the effect of more daylight for feeding since. during hyperphagia, the amount of daylight increases only 9%. The temporary hyperphagia is the principal factor responsible for positive energy balance required for premigratory fat deposition. The role of daily photoperiod in development of temporary hyperphagia was demonstrated experimentally by subjecting birds in mid-winter to 20 hours of light per day. These developed a hyperphagia and an accompanying fat deposition very similar to those observed in spring under natural conditions.

We wish to acknowledge the assistance of Dr. L. R. Mewaldt in obtaining some of the lipid indices reported herein.

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1. Wolfson, A., *Condor*, 1942, v44, 237.
2. ———, *ibid.*, 1953, v55, 187.
3. ———, *J. Exp. Zool.*, 1954, v125, 353.
4. Schildmacher, H., and Steubing, L., *Biol. Zrbl.*, 1952, v71, 272.
5. Koch, H. J., and de Bont, A. F., *Ann. Soc. Roy. Zool. Belgique*, 1952, v82, 1.
6. Odum, E. P., and Major, J. C., *Condor*, 1956, v58, 222.
7. Wallgren, H., *Acta Zool. Fenn.*, 1954, v84, 1.
8. Benoit, J., and Assenmacher, I., *J. Physiol.*

(Paris), 1955, v47, 427.

9. Hetherington, A. W., and Ranson, S. W., *Anat. Rec.*, 1940, v78, 149.

10. Anand, B. K., and Brobeck, J. R., *Yale J. Biol. Med.*, 1951, v24, 123.

11. Mayer, J., *Ann. N. Y. Acad. Sci.*, 1955, v63 (art. 1), 15.

12. Groebbels, F., *Pflügers Arch.*, 1928, v218, 98.

13. Merkel, F. W., *Zool. Anz.*, 1937, v117, 297.

14. Kendeigh, S. C., *Auk*, 1949, v66, 113.

15. McGreal, R. D., Master's Thesis, State Coll. of Washington, Pullman, 1954.

16. McGreal, R. D., and Farner, D. S., *Northwest Sci.*, 1956, v30, 12.

17. Brody, S., *Bioenergetics and Growth*, p34, Rheinhold Press, New York, 1945.

18. Schwabe, E. L., and Griffeth, F. R., Jr., *J. Nutrition*, 1938, v15, 178.

19. Benedict, F. W., *Carnegie Inst. Wash.*, Publ. 503, 1938.

20. Farner, D. S., and Mewaldt, L. R., *Bird-Banding*, 1953, v24, 55.

21. Farner, D. S., Mewaldt, L. R., and King, J. R., *J. Comp. Physiol. Psych.*, 1954, v47, 148.

22. Odum, E. P., and Perkinson, J. D., Jr., *Physiol. Zool.*, 1951, v24, 216.

23. Odum, E. P., and Connell, C. E., *Science*, 1956, v123, 892.

24. Farner, D. S., *Recent Studies in Avian Biology*, 1955, pp. 198-237, Univ. Ill. Press, Urbana, 1955.

25. Miller, A. H., *Condor*, 1954, v55, 13.

26. Farner D. S., and Mewaldt, L. R., *ibid.*, 1955, v57, 112.

27. Seibert, H. C., *Auk*, 1949, v66, 128.

28. Davis, E. A., Jr., *ibid.*, 1955, v72, 385.

29. Bergman, G., *Ornis Fennica*, 1950, v27, 109.

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Glycogen Deposition in Squamous Metaplastic Epithelium of the Rat Uterus.* (22756)

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The deposition of glycogen in the normal uterus of the rat has been studied by both chemical(1) and histochemical methods(2). It has been demonstrated by chemical analysis that the total glycogen content of the rat uterus varies with the stage of the estrous cycle, being maximal during proestrus and minimal during diestrus. Histochemically, it has recently been shown that the glycogen of the rat uterus is limited to the muscle cells of the myometrium; in the proestrous animal the material is abundant throughout the longitudinal layer but is irregularly distributed in the circular layer while in the diestrous uterus, the glycogen content of the longitudinal muscle layer is greatly reduced and that of the circular muscle layer has disappeared entirely.

The distribution of glycogen in abnormal uteri, particularly in epithelial metaplasia of the rat uterus has not been reported. One of the methods used to produce and study uterine metaplasia in the intact rat is by placing the animal on a vit. A deficient diet(3). Using this method to produce epithelial metaplasia, the present investigation was undertaken to study, by the periodic acid-leucofuchsin technic, the distribution of glycogen in the metaplastic uterine glands of the intact rat.

Materials and methods. Twenty-five rats of the Wistar strain were used in these experiments. The animals were 20 to 22 days of age and weighed approximately 35 to 40 g when placed on the vit. A free diet.† The rats were killed with chloroform during the 9th to 14th week of the deficiency and the uteri were fixed immediately. A portion of each uterus

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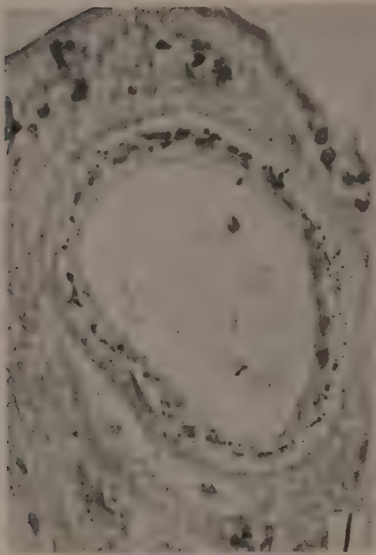
† Vitamin A Test Diet U.S.P., Nutritional Biochemicals Corp., Cleveland, O.



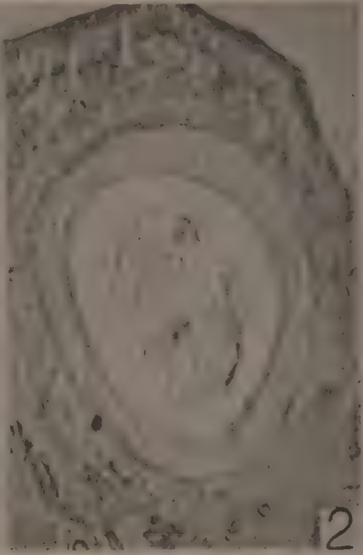


FIG. 1. Photomicrograph of a metaplastic gland following vit. A deficiency. The section was stained by the periodic acid-leucofuchsin method. Note dark glycogen granules in epithelium of metaplastic gland. × 410.

FIG. 2. Photomicrograph of a control section, close to that illustrated in Fig. 1, which was incubated in malt diastase to remove glycogen before staining. Note absence of glycogen granules from epithelium of metaplastic gland. Residual color in the section is due to staining of mucopolysaccharides and glycoprotein. × 410.

was fixed in cold modified PAF (10% formalin in 80% ethyl alcohol saturated with picric acid), cold acetone and in 10% formalin containing 2% calcium acetate. The present report is concerned with the study of glycogen deposition in epithelial metaplasia of the uterus following vit. A deficiency. After fixation in PAF the tissue was dehydrated, embedded in paraffin and sections were cut 12 μ in thickness. These were treated with periodic acid and then stained with leucofuchsin (4). Both glycogen and mucopolysaccharides are stained a reddish purple. In order to distinguish between them, control slides were incubated for an hour in 1% malt diastase buffered at pH 6.8 to remove the glycogen before applying the periodic acid-leucofuchsin procedure. No counterstain was used.

Observations. In the metaplastic glands that were observed in the uteri of the vit. A deficient animals, PAS positive material was present in the cells of the stratified epithelium. The granules were present primarily in the intermediate layer of the epithelium. In the control sections that were treated with 1% malt diastase, the material was not present indicating the presence of glycogen (Fig. 1 and 2).

The secretory product of the luminal and glandular epithelium that did not show any metaplastic changes was PAS positive but was resistant to 1% malt diastase which indicated that the material was not glycogen but a mucopolysaccharide.

The amount of glycogen observed in the tunica muscularis was variable; none was observed in the majority of the animals. In some of the animals only a trace of glycogen was observed in the longitudinal layer. The distribution of the glycogen in the uterine muscle was similar to that previously reported in the castrate and diestrous rat(2).

Discussion. The normal epithelium of the rat uterus, as was reported previously(2), does not contain any glycogen. However, the present results show that in the metaplastic epithelium following vit. A deficiency, glycogen granules are present in the cells of the new epithelium.

The significance of glycogen deposition in squamous metaplasia of the uterus following vit. A deficiency and the biochemical enzyme systems involved is not known at the present time. There is evidence that the most important single factor which determines the rate of mitosis in tissue, such as mammalian epidermis, is the energy supply within the cells which depends upon the intracellular availability of glucose derivatives(5). If it can be assumed that because of increased mitotic activity the energy requirements of metaplastic epithelial cells is greater than for normal uterine epithelial cells, then the glycogen present in the metaplastic cells may be a source of readily utilizable energy.

The present results on glycogen deposition in epithelial metaplasia differs from the observation made in the human(6). In one instance a single squamous metaplastic cervical gland of one patient was reported to be free of glycogen. Since there appears to be a difference in glycogen deposition in squamous metaplasia, investigations are under way to determine whether the deposition of glycogen in stratified squamous metaplasia is peculiar to the changes following vit. A deficiency or whether the deposition of glycogen occurs in all epithelial metaplasia.

Summary. In the epithelial metaplasia of the rat uterus that occurred following vit. A deficiency, glycogen was observed in the stratified epithelium. An earlier report showed that the normal uterine epithelium of the rat uterus contains no glycogen.

1. Boettiger, E. G., *J. Cell. and Comp. Physiol.*, 1946, v27, 9.
2. Bo, W. J., and Atkinson, W. B., *Anat. Rec.*, 1952, v113, 91.
3. Wolbach, S. B., and Howe, P. R., *J. Exp. Med.*, 1925, v42, 753.
4. McManus, J. F. A., *Am. J. Path.*, 1948, v24, 643.
5. Bullough, W. S., *Vitamins and Hormones*, Academic Press, Inc., New York, 1955, v8, 261.
6. McManus, J. F. A., and Findley, L., *Surg. Gynec. and Obst.*, 1949, v89, 616.

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Effect of Methotrexate Therapy upon Choriocarcinoma and Chorioadenoma. (22757)

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Folic acid is known to be essential for the growth of the female genital tract and for normal embryonic development. Hertz *et al.* demonstrated the inhibition of estrogen-induced growth in the chick oviduct and monkey uterus in animals maintained on a folic acid deficient diet or treated with antagonists of either folic acid or adenine(1,2,3). Reduction of a previously high titre of gonadotropin occurred following a short course of methotrexate in a hypophysectomized woman with metastatic melanoma.*

The present report includes observations on the effect of a repeatedly administered and intensive regimen of methotrexate upon the urinary excretion of chorionic gonadotrophin and upon the clinical course in 2 patients with choriocarcinoma and one patient with chorioadenoma destruens.

Methods. The diagnosis was established by histological evidence. The initial extent of the disease was determined by physical examination and by radiological survey. Other observations included recording on each day of (a) fluid intake and output, (b) caloric intake, (c) the hemogram, and (d) the body weight. Each week x-rays were taken and measurement of palpable or visible lesions was recorded. Urinary gonadotropin determinations were carried out by a modification of the method of Klinefelter *et al.*(4). Gonadotropin values are expressed as "mouse uterine units" per 24 hours. The titres on

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* Unpublished.

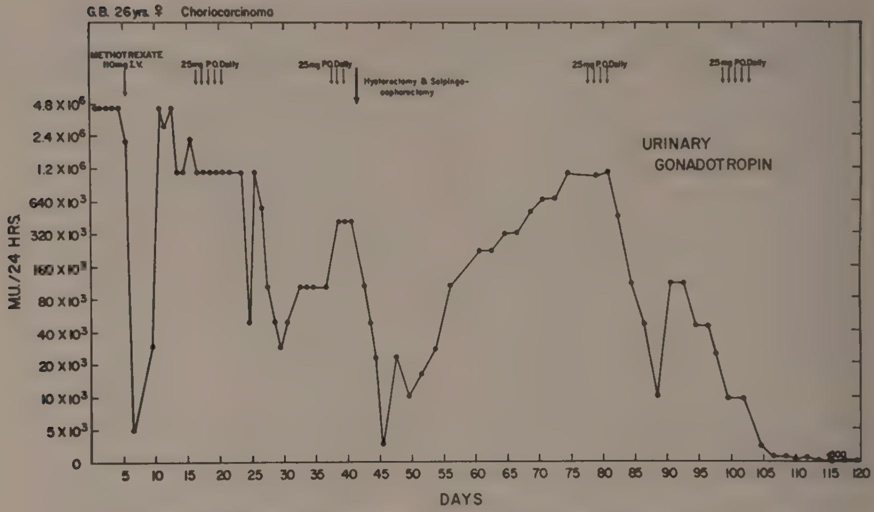

FIG. 1. Effect of intensive methotrexate therapy on urinary gonadotropin: Case 1.

graphs represent highest dilution yielding a positive reaction and twice this dilution was usually found to be negative. *Therapeutic Regimen*: The dosage regimen† of methotrexate is shown in the accompanying charts (Fig. 1, 2 and 3.) Two weeks after an initial single intravenous dose of 1 to 4.5 mg/kg body weight, there is given repeatedly at about 2 week intervals additional courses of 2.5 mg/kg body weight divided in 5 consecutive daily doses. The final course was given after the urinary gonadotropin titre had fallen to levels approaching normal with a view to a more sustained effect. Daily appraisal of the patient's hemogram, renal function, and clinical status with respect to stomatitis, glossitis, diarrhea, proctitis, and petechiae preceded the administration of the next dose. New courses were started only after clear evidence of complete hematological and gastrointestinal recovery from previous toxic effects.

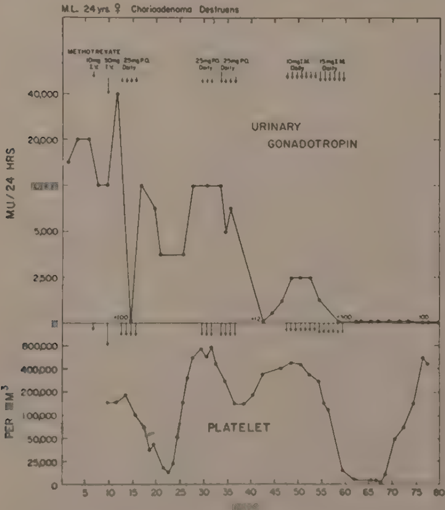

FIG. 2. Same as Fig. 1, and circulating platelet count: Case 3.

Results. By close adherence to the above mentioned precautions no undue toxicity was noted in these 3 patients. Experience with this same regimen (Fig. 3) emphasizes the possibility of renal impairment and this should be carefully checked by frequent BUN determinations and urinalyses. Moreover, preexisting renal impairment would constitute a definite contra-indication to the use of this regimen especially since any renal impairment would delay excretion and thereby enhance toxicity. Hepatic injury has not been observed.

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† This regimen represents modification of procedure for intensive methotrexate administration devised by Dr. Paul T. Condit, Clinical Pharmacol. and Exp. Therap. Section, National Cancer Institute.

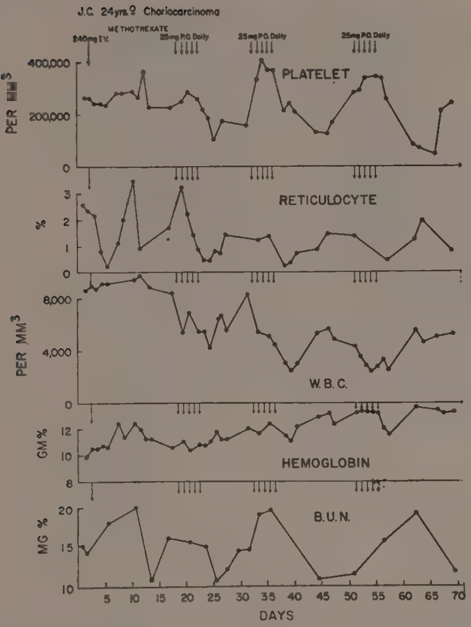

FIG. 3. Effect of intensive methotrexate therapy on the homopoietic elements and renal function: Case 2.

Fig. 2 and 3 illustrate transient impairment of hemopoiesis with each course of methotrexate. These effects began toward the close of each course and became most marked on the 4th to 10th day but full recovery was usually noted within 14 days.

Gastrointestinal difficulties such as anorexia, nausea, diarrhea, stomatitis, glossitis, ulceration of oral and rectal mucosa usually appeared and receded at about the same time as the bone marrow effects. These evidences of toxicity appeared to become progressively more apparent with each course of therapy.

Case 1. G.B., 26-year-old white housewife. She had a large pelvic mass and multiple bilateral pulmonary metastases. Diagnosis of choriocarcinoma had been established on tissue obtained by curettage. This case had been accepted for registration as choriocarcinoma in the Mathieu collection. On admission the patient had marked anorexia, protracted vomiting, and repeated bouts of uterine hemorrhage. There was evidence of marked weight loss and general debility. The urinary gonadotropin assay was positive at 4,000,000 mouse units/24 hours. The 5 courses of methotrexate given are indicated in Fig. 1. During the third course of methotrexate uterine bleeding became sufficiently profuse to indicate an emergency hysterectomy and salpingo-oophorectomy. Recovery was uneventful and further methotrexate therapy became indicated when the urinary gonadotropin titre had returned in a 6 weeks period from an immediate post-operative titre of 2,500 mouse units to 1,000,000 mouse units. After each course of methotrexate the gonadotropin titre was markedly suppressed (Fig. 1). With each successive course both the degree and duration of the gonadotropin reduction became greater. Following the 5th course the titre had receded to below 500 mouse units and has now remained at this reduced level for 5 weeks.

Correlated with this recession in urinary gonadotropin titre there was noted unequivocal x-ray evidence of regression of the pulmonary lesions (Fig. 4) and distinct amelioration of the patient's general clinical status.

Case 2. J.C., 24-year-old white housewife. She had a large, fixed, and irregular pelvic mass extending to the level of the umbilicus. She also had a globoid mass measuring 3 x 4 cm arising in the left labium major. Chest x-ray revealed diffuse bilateral pulmonary metastases. There had been extended vaginal hemorrhage and the patient was severely debilitated and critically ill. Several biopsy specimens showed choriocarcinoma with large masses of neoplastic cells presumably of trophoblastic origin. No villi were noted. The urinary gonadotropin titre was 200,000 mouse units/24 hours. Patient received 4 successive courses of methotrexate therapy. After the 4th course of therapy the gonadotropin titre had receded to below 200 mouse units and has remained at this level for the ensuing 6 weeks.

Accompanying this recession in gonadotropin titre the pelvic mass decreased to half its initial size and the labial mass was completely resolved. X-rays revealed striking clearing of the pulmonary lesions. The patient was completely rehabilitated and re-

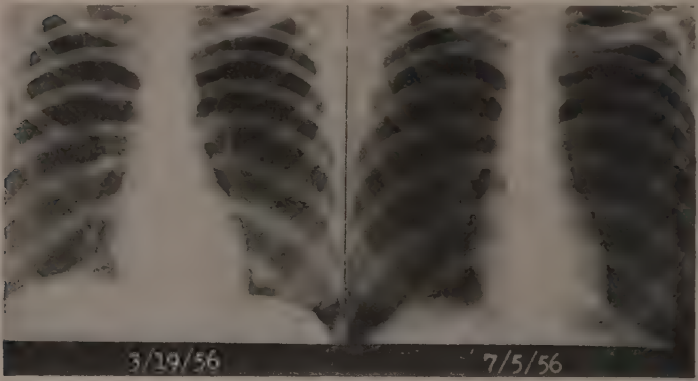

FIG. 4. Regression of pulmonary metastases. Case 1. Methotrexate therapy initiated 3/12/56.

mains under observation at this time on an ambulatory basis.

Case 3. M.L., 24-year-old housewife. Six months prior to admission she had undergone hysterectomy and bilateral salpingo-oophorectomy. The confirmed histological diagnosis was chorioadenoma destruens. On admission she was critically ill due to rapid progression of radiologically demonstrable pulmonary metastases. The urinary gonadotropin titre was 20,000 mouse units/24 hours.

Fig. 2 presents the therapeutic regimen and the associated changes in gonadotropin excretion in this case. It will be noted that the titre had dropped to below 300 mouse units and the patient experienced complete clinical recovery. During the past 8 months her gonadotropin titre has been found at monthly intervals to range between 100 and 200 mouse units. This titre is normal in our laboratory for an ovariectomized patient. Monthly x-rays (Fig. 5) show no evidence of metastatic disease since March this year.

Discussion. The correlation between clinical course and histopathological findings is notoriously confused in the case of trophoblastic tumors. Novak(5) and numerous others have described the pitfalls attendant upon clinical prognostication in this group of diseases on the basis of even the most expert histopathological analysis. Accordingly, we do not propose to draw any definitive therapeutic conclusions on the basis of the limited observations reported here. Nevertheless, the association of the as yet benign clinical course of these 3 patients with the methotrexate-induced recessions of urinary gonadotropin titre and regression of pulmonary metastases indicate the desirability of more extended study of the therapeutic potentialities of intensive treatment with antifolic and related compounds in this group of diseases.

Thiersch and Phillips(6) observed that the placental tissue in aminopterin-treated rats were not especially affected. This brings up the question whether methotrexate therapy employed here may have more general clinical application than in the type of cases just described. However, previous clinical experience with less intensive anti-folic regimens in several types of neoplasms other than acute leukaemia has not been encouraging(7). In any event, these observations emphasize the experimental advantage of an available index of tumor activity such as gonadotropin excretion in evaluation of therapeutic agents.

The intensive regimen of methotrexate therapy with courses interposed between successive periods of recovery from gastrointestinal and hematological toxicity is predicated upon the expected reversibility of such effects in normal tissues and possible failure of such reversibility in tumor tissue after repeated courses. The behavior of the urinary gonadotropin titres in these cases suggests that this expectation may have been realized. More prolonged follow-up in more cases will be required for further evaluation of such a rationale.

Caution was taken to ascertain whether the

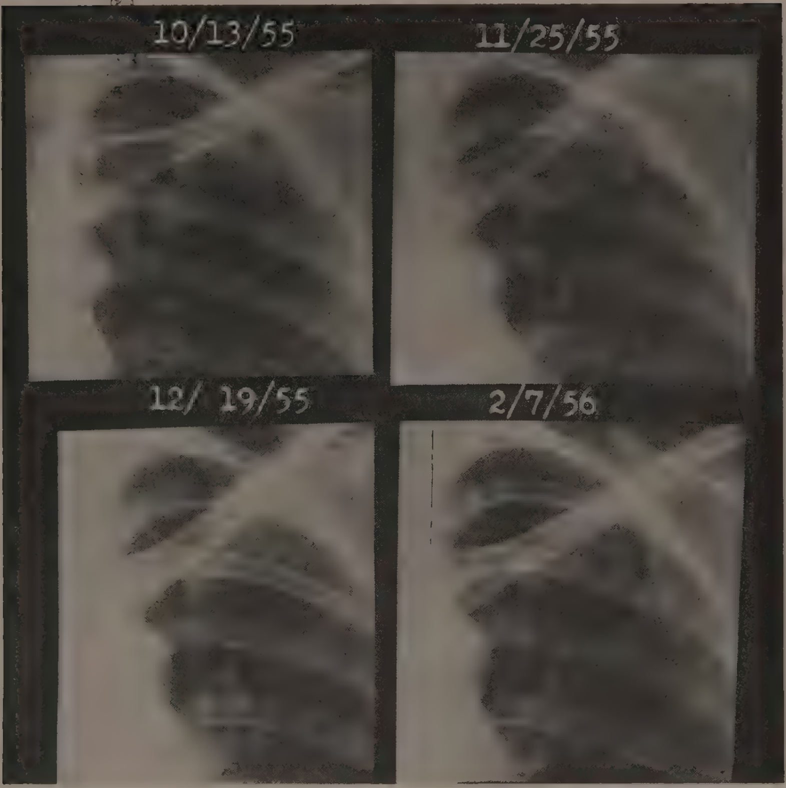

FIG. 5. Regression of pulmonary metastases. Case 3. Methotrexate therapy initiated 10/20/55.

apparent reduction in urinary gonadotropin may not have resulted from interference of excreted methotrexate and its metabolites with the biological assay. Addition of methotrexate to high titre urines did not alter the titre obtained probably because the dialysis of the urinary concentrate effectively removed substances of small molecular weight. Moreover, the persistence of the reduced titres for prolonged periods following cessation of therapy would render the possibility of such chemical interference unlikely.

Summary. 1) Two patients with choriocarcinoma and one with chorioadenoma destruens with proven progressive metastases were treated with methotrexate, a folic acid antagonist, according to intensive regimen of repeated courses of dosages producing substantial but reversible toxicity. Initially high urinary gonadotropin present in all 3 cases approached normal levels following this regimen and such low levels were sustained following cessation of treatment. 2) Unequivocal clinical improvement and radiological evidence of regression of pulmonary metastases was observed in all 3 cases. Nevertheless, extreme variations in pattern of clinical behavior of this type of tumor necessitate more observations before any definitive therapeutic implications may be drawn from these studies.

We are deeply grateful to Dr. Delbert M. Bergenstal, to Dr. Gordon Zubrod, and to Dr. Herbert A. Lubs for their valuable criticisms.

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1. Hertz, R., and Sebrell, W. H., *Science,* 1944, v100, 293.

2. Hertz, R., and Tullner, W. W., *Endocrinology,* 1949, v44, 278.

3. Hertz, R., *Science,* 1948, v107, 300.

4. Klinefelter, H. F., Jr., Albright, F., and Griswald,

G. C., *J. Clin. Endocrinol.*, 1943, v3, 529.
5. Novak, E., and Seah, C. S., *Am. J. Obst. and Gynec.*, 1954, v67, 933.
6. Thiersch, J. B., and Phillips, F. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 204.
7. Burchenal, J. H., Karnofsky, D. A., Kingsley-Pillers, E. M., Southam, C. M., Myers, W. P. L., Escher, G. C., Craver, L. G., Dargeon, H. W., and Rhoads, C. P., *Cancer*, 1951, v4, 549.

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Anti-Inflammatory Protection of Guinea Pigs by Egg Yolk: Effect on Adrenals and Serum Constituents. (22758)

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Coburn *et al.*(3) have shown that dietary egg yolk reduces the intensity of the inflammatory response induced by passive anaphylactic arthritis in young guinea pigs. Their observations indicated that the active material of egg yolk resides in the alcohol soluble (crude phosphatide) fraction and is absent from both the acetone solubles (neutral fats and cholesterol) and the yolk protein. We have confirmed the above findings(19) using the anti-inflammatory bio-assay method of French *et al.*(8). The adrenal hormone, cortisone, and its related steroids have been used in treatment of inflammatory diseases(15,18). Their protection of laboratory animals against inflammation induced by BCG(21), tuberculin(2), turpentine(16), and Pneumococci(22) has also been demonstrated. French *et al.* (8) showed that cortisone treatment also reduced swelling in joints of guinea pigs which had been subjected to a reverse Arthus reaction.

The present investigation was undertaken (a) to compare the anti-inflammatory protection by cortisone to that afforded by dietary egg yolk, (b) to study the effect of yolk alcohol solubles on adrenals of the guinea pig, and (c) the effect of egg yolk on blood constituents of guinea pigs.

Methods. 1. *Fractionation of egg yolk powder.*† Acetone solubles, alcohol solubles and the protein fraction of egg yolk were obtained by procedures of Coburn *et al.*(3). 2. *Assaying procedure.* Weanling guinea pigs weighing about 160 g (unless otherwise indicated) were used as test animals in groups of 6 or more. Each animal was weighed just prior to, and every 3 days during the experiment, and daily food intake was recorded. Egg yolk and yolk fractions were individually fed to guinea pigs as supplements to a basal control diet for a period of 21 to 26 days. The basal diet contained 50% Rockland guinea pig diet and 50% dixie rabbit pellets (22% protein) to which was added 0.1% ascorbic acid. At end of the feeding period, the guinea pigs usually reached the desired weight of approximately 300 g and then used for bio-assay(8). For each assay group, a control group of 6 or more guinea pigs was fed and challenged at the same time to minimize environmental influences on inflammation.

Results. *Exp. 1* was a comparison of the anti-inflammatory activity of egg yolk and yolk alcohol solubles to that of cortisone. Cortisone acetate was injected intraperitoneally into 300 g guinea pigs (after 3-4 days on basal diet) 1 hr prior to assay. For each assay a second group of guinea pigs, pretreated with saline, was used as control. Feeding dried egg yolk at 19.5%, or yolk alcohol solubles at 2-8% of diet, resulted in 28-40% inhibition of swelling (Table I) similar to that obtained by administering cortisone acetate at the level of 25 mg/kg body weight(8). When alcohol solubles were fed at less than 2% of diet, no protection was observed.

Exp. 2 was a study of the effect of dietary yolk alcohol solubles on adrenals of the guinea

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† Prepared by Wilson Laboratories, Chicago, Ill.

TABLE I. Effect of Feeding Dried Egg Yolk Powder and Yolk Alcohol Solubles on Mean Swelling Index (M.S.I.) in Reverse Arthus Reaction of Guinea Pigs.

No. guinea pigs	Fed control, M.S.I. ± S.E.	No. guinea pigs	Yolk powder, 19.5%	Yolk alcohol solubles 1%	2%	4%	6%	8%	% inhibition
				M.S.I. ± S.E.					
6	4.8 ± .37	8	3.4 ± .37						28
7	4.8 ± .34	6		5.6 ± .43					0
6	6.0 ± .30	5			4.3 ± .59				28
6	4.0 ± .37	7				3.0 ± .53			25
6	6.0 ± .30	6					3.9 ± .77		35
7	4.6 ± .41	6						3.4 ± .18	26
6	4.8 ± .31	6						2.9 ± .20	40
6	5.2 ± .50	7						3.1 ± .50	40

pig. Guinea pigs weighing 160 or 200 g were fed for periods of 21 and 10 days respectively. At completion of the assay, the guinea pigs were killed by blow on head. The adrenals were quickly removed, cleaned from adherent tissues, washed with physiological saline, weighed and frozen. They were kept in frozen state until needed. The adrenals in each group were pooled and water content was determined(1). The total lipid content was determined on ground, dried adrenals by extraction with hot chloroform and methanol (2:1) in Soxhlet extractor for 36 hrs. The extract was evaporated to dryness in water bath at 40°C under stream of nitrogen. The residue was redissolved in chloroform at room temperature and filtered. The total lipid was calculated from the weight of residue remaining after filtrate was dried to a constant weight. The total cholesterol in lipid extract was then determined(23).

The similarity between the anti-inflammatory protection afforded the guinea pig by dietary egg yolk and by injected cortisone acetate suggested that the egg yolk effect might be mediated by the adrenals. However, no indication of increased adrenal hormone production was observed in the treated guinea pigs (Table II). Adrenal weights were not increased nor were adrenal cholesterol levels depressed by feeding yolk alcohol solubles.

Exp. 3. The effect of dietary egg yolk on certain blood constituents of guinea pigs was studied. Serum lipids of guinea pigs, fed egg yolk, or 1 of 3 egg yolk fractions (alcohol solubles, acetone solubles and protein fraction) were determined. Each experimental diet contained one or another of the 3 fractions in a quantity equivalent to that which would be found if 19.5% of diet weight were egg yolk. In studying the effect of egg yolk on serum total N and non-protein N levels, the guinea pigs were fed the alcohol soluble fraction. Heart blood samples were obtained from fasting animals 24 hrs after completion of the assay. Total lipid was determined by extracting lyophilized serum with hot chloroform and methanol (2:1) as described for determination of adrenal lipid content. Lipid phosphorus and serum cholesterol were then determined(11,23). Phospholipid values were calculated by multiplying lipid phosphorus by 26. The serum total N and non-protein N of the protein-free filtrate(7) were determined by micro-Kjeldahl(13,14). No essential dif-

TABLE II. Effect of Yolk Alcohol Solubles on Adrenals of 6 Guinea Pigs in Each Series.

Treatment	Days	Wt, mg/100 g body wt	Water, %	Lipid, %*	Cholesterol, %†
Control	21	46.5 ± 4.5	57.1	50.5	25.3
Yolk alcohol solubles, 8%		38.3 ± 2.8	56.6	48.2	25.6
Control	10	37.1 ± 4.1	65.6	54.6	17.2
Yolk alcohol solubles, 2%		42.5 ± 3.5	63.7	47.2	20.7
" " " , 6%		42.3 ± 2.8	63.6	49.6	20.1

* Expressed as % of dry wt of adrenals.
† " " " " lipid content of adrenals.

TABLE III. Serum Total Lipid, Phospholipid and Cholesterol Levels of Guinea Pigs on Control and Supplemented Diets.

Treatment	Total lipid, mg/ml	P	Phospholipid, mg/ml	P	Total cholesterol, mg/ml	P	C/P
Control	2.03 ± .12*		.384 ± .04*		.466 ± .07*		1.21
Dried yolk powder	4.79 ± .92	.0125	.483 ± .08	>.1	.606 ± .08	>.1	1.25
Control	2.14 ± .22		.324 ± .06		.258 ± .07		.80
Yolk protein	14.60 ±1.17	.0025	3.774 ± .42	<.0025	7.410 ± .34	<.0025	1.96
Control	2.07 ± .22		.376 ± .04		.437 ± .05		1.16
Yolk alcohol solubles	3.55 ± .71	.025	.533 ± .05	.05	.468 ± .03	>.1	0.91
Control	2.78 ± .09		.406 ± .04		.354 ± .02		0.87
Yolk acetone solubles	4.42 ± .80	.025	1.074 ± .29	.025	1.046 ± .31	<.025	0.97

* ± S.E.

ference was found in average daily food intake and weight gain between guinea pigs fed control or supplemented diets. Animals, which were fed yolk alcohol solubles, received the same protection as those fed a supplement of dried yolk powder. Both the protein and the acetone soluble fractions appeared inactive.

Guinea pigs which had received acetone soluble material possessed enlarged fatty livers and gallbladders. Guinea pigs from other groups had normal organs.

Feeding dried egg yolk and yolk alcohol-solubles resulted in elevation of serum total lipid with no increase in cholesterol to phospholipid ratio (Table III). Feeding inactive yolk fractions caused elevation of serum total lipids, phospholipids, cholesterol, and the cholesterol to phospholipid ratio. The serum total N and non-protein N were unaffected by feeding yolk alcohol solubles.

Discussion. An anti-inflammatory effect of phospholipid, sphingomyelin has been demonstrated by several workers. Kerr *et al.*(12) showed that sphingosine, as well as sphingomyelin, inhibited the skin reaction in cattle sensitized to *Trichomonas foetus* antigen. The favorable effect of sphingomyelin and sphingosine has also been observed in guinea pigs sensitized to tuberculin(6).

In preliminary fractionation of alcohol solubles of egg yolk, we found that the active factor was present in the remaining fraction of alcohol solubles, after lecithin had been precipitated. The lecithin fraction which was obtained by a cadmium chloride precipitation did not appear to be active. The possibility that the active yolk material is also associated with the crude sphingomyelin fraction has been suggested by Coburn *et al.*(3).

Of the serum constituents studied, the total lipid fraction was elevated by feeding egg yolk or yolk alcohol solubles. Serum phospholipids and cholesterol were slightly elevated by these two diet supplements. Therefore, it seems that elevation of certain serum lipids other than cholesterol and phospholipids accounts for the hyperlipemia observed after feeding active egg yolk fractions. Hyperlipemia produced by active yolk material is quite comparable to that observed in rat, rabbit and human after administration of cortisone(5,4,10,20). These reports indicated that administration of cortisone caused hyperlipemia due chiefly to increase of lipids other than cholesterol and phospholipids. The inactive yolk fractions both caused an increase in serum total lipids proportional to the increase of phospholipids and cholesterol. If the inhibitory effect of the active factor on the experimental inflammation in guinea pigs depends on changes in the blood lipid fractions, then it seems logical to attribute its effect to the associated increase in lipid fractions other than cholesterol and phospholipids. However, the possibility that active yolk material inhibits induced inflammation independent of its effect on blood lipids cannot be excluded at present.

Summary. The anti-inflammatory activity of egg yolk has been studied in weanling

guinea pigs. 1. The active material resided in the alcohol soluble fraction and was absent from the protein and acetone soluble fractions. 2. The anti-inflammatory effect did not appear to be mediated by the adrenals. 3. Feeding egg yolk and yolk alcohol solubles resulted in an elevation of serum total lipids with no increase in cholesterol to phospholipid ratio. Inactive yolk fractions caused elevations of serum total lipids, phospholipids, cholesterol, and cholesterol to phospholipid ratio. 4. Serum levels of total N and non-protein N were unaffected by yolk alcohol solubles.

The authors wish to express their thanks to Drs. John R. Allen and Joseph C. Street for their assistance in preparing egg yolk fractions and conducting the anti-inflammatory assays.

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1. Assn. of Official Agricultural Chemists, *Official and Tentative Method of Analysis*, 7th ed., Washington, D. C., 1950,

2. Birkhang, K., *Ann. Rev. Tuberc.*, 1954, v69, 511.

3. Coburn, A. F., Graham, C. E., and Haninger, J., *J. Exp. Med.*, 1954, v100, 425.

4. Dury, A., *Am. J. Med. Sci.*, 1955, v203, 427.

5. Dury, A., and DiLuzio, N. R., *Am. J. Physiol.*, 1955, v182, 45.

6. Fisher, N., Harrington, C., and Long, D. A., *Lancet*, 1951, vVII, 522.

7. Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, v38, 81.

8. French, C. E., Allen, J. R., and Davis, K. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 41.

9. Germuth, F. G., Jr., and Ottinger, B., *ibid.*, 1950, v74, 815.

10. Hansted, C., and Anderson, H., *Acta Paediat.*, 1953, v42, 386.

11. Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical Physiological Chemistry*, 12th Ed., Philadelphia, The Blakiston Co., 1947, p540.

12. Kerr, W. R., and Robertson, M., *J. Hygiene*, 1952, v50, 354.

13. Keys, A., *J. Biol. Chem.*, 1940, v132, 181.

14. Ma, T. S., and Zuazaga, G., *J. Ind. and Eng. Chem.*, (Anal. Ed.) 1942, v14, 280.

15. McIntire, F. C., Roth, L. W., and Sproull, M., *Am. J. Physiol.*, 1951, v167, 233.

16. Menkin, V., *Sci.*, 1954, v120, 1026.

17. Pula, D., and Marmorston, J., 1941, *Natural Resistance and Clinical Medicine*, Chap. 18, Boston, Little Brown & Co.

18. Selye, H., *The Physiology and Pathology of Exposure to Stress*, Acta, Montreal, Canada, 1950.

19. Street, J. C., Allen, J. R., Chang, I., and French, C. E., Am. Chem. Soc. 128th Meeting, 1955, 32C.

20. Stumpf, H. H., and Wilena, S. L., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 219.

21. Ungar, G., Damgaard, E., and Weinstein, H. G., *Am. J. Physiol.*, 1951, v166, 340.

22. Vollmer, E. P., *J. Infect. Dis.*, 1951, v88, 27.

23. Zlatkis, A., Zak, B., and Boyle, A. J., *J. Lab. Clin. Med.*, 1953, v41, 486.

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Δ^1-Hydrocortisone and Hydrocortisone: Plasma 17-Hydroxycorticosteroid Concentrations in the Dog Following Oral and Intravenous Administration.

(22759)

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Δ^1-Hydrocortisone ($\Delta^{1,4}$-pregnadiene-11β, 17α, 21-triol-3,20-dione) has received considerable attention as a potent synthetic analogue of hydrocortisone (Δ^4-pregnene-11β, 17α, 21-triol-3, 20-dione). Clinical studies have indicated Δ^1-hydrocortisone to be 3 to 5 times as potent as the parent steroid(1-3). Evaluation of glucocorticoid activity (liver glycogen deposition, eosinopenic response, thymus involution) has shown a similar ratio (4). Introduction of unsaturation at the C_1 position of the steroid A-ring could conceivably alter the rates of absorption and/or metabolism of the compound. In the present study, this possibility has been examined experimentally in the dog by determining the changes in plasma 17-hydroxycorticosteroid (17-OHCS) concentrations as a function of time following oral and intravenous administration of Δ^1-hydrocortisone and hydrocortisone. Subsequent to this study, a similar evaluation of Δ^1-hydrocortisone in the human has been reported(5).

Materials and methods. Male mongrel

dogs weighing 15-20 kg were used in these studies. The animals were fasted for 16-18 hours prior to the steroid administration and food but not water was withheld during the blood sampling period. In experiments involving oral administration, the preparations tested were as follows: (a) Δ^1-hydrocortisone: 5 mg (5 expts), 20 mg (4 expts), and 40 mg (4 expts); (b) hydrocortisone: 10 mg (4 expts), 20 mg (23 expts), and 40 mg (4 expts). Following administration of the compressed tablets, 3-6 blood samples (25-45 ml) were collected over periods of 2-5 hours from the external jugular vein without anesthesia. For the intravenous studies, the steroids were administered as the free alcohols (1 mg/ml in 10% ethanol-isotonic saline) and as the 21-hemisuccinate sodium salts (equivalent to 1 mg of free steroid/ml in isotonic saline) by a rapid infusion into the antecubital vein at a dose of 2 mg/kg. During a 3-4 hour period, 7-8 blood samples were collected. Plasma 17-OHCS concentrations were determined by a modification of the Nelson-Samuels procedure(6,7,8). In order to avoid frequent occurrence of cloudy solutions in the spectrophotometric step, it was necessary to partition the residue from the chloroform extract of dog plasma between 70% ethanol and Skellysolve B. The 70% ethanol extractables were then carried through Florisil chromatography as described by Nelson and Samuels(6). The spectrophotometric procedure was modified to include a direct correction for non-specific, acid-produced chromogens(8) similar to that originally recommended by Porter and Silber (9).

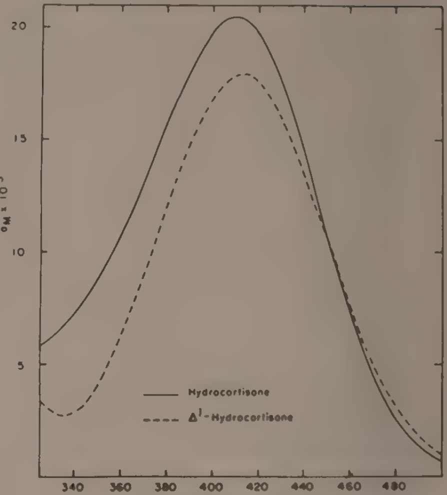

FIG. 1. Absorption spectra of phenylhydrazine-sulfuric acid chromogens from Δ^1-hydrocortisone and hydrocortisone (a_M calculated on basis of steroid conc. in reaction mixture).

Results. Before applying routine 17-OHCS procedures in studies involving Δ^1-hydrocortisone, it was necessary to know the reactivity of this steroid with the phenylhydrazine-sulfuric acid reagent as well as its behavior on Florisil columns. The absorption spectra of the phenylhydrazine-sulfuric acid chromogens obtained from Δ^1-hydrocortisone and hydrocortisone are shown in Fig. 1. Absorptivity at the maximum is less for Δ^1-hydrocortisone than for hydrocortisone and there is a slight shift of the maximum toward longer wavelengths due to the effect of the unsaturation at C_1. Routine measurements for both steroids were made at 410 mμ. By the procedure employed(8), the calibration curves at this wave length obey Beer's law over a wide range, and Δ^1-hydrocortisone is 87% as chromogenic as hydrocortisone. Recovery of Δ^1-hydrocortisone from Florisil columns was slightly but consistently less than hydrocortisone. With a typical batch of Florisil, recoveries (mean ± s.e.m.) were as follows: Δ^1-hydrocortisone, 88.1 ± 1.1% (35 samples); hydrocortisone, 90.4 ± 1.4% (14 samples). For these reasons, the steroid administered was used as reference standard for chromatography and spectrophotometry.

To interpret data following intravenous administration of the steroid hemisuccinates, the behavior of hydrocortisone-21-hemisuccinate during Florisil chromatography was examined. None of the ester was eluted by either 2% or 25% methanol-in-chloroform. Further elution with 40% methanol-in-chloroform recovered about 30% of the ester. Therefore, even though some of the ester was extracted from plasma, it was removed during chromatography and only free 17-OHCS were measured. Previous studies have shown that neutral esters such as the acetate and cyclopentylpro-

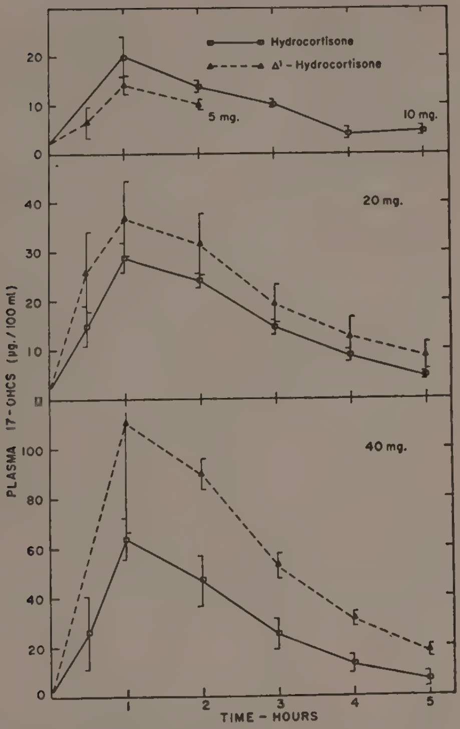

FIG. 2. Plasma 17-OHCS conc. (mean ± S.E.) vs. time following oral admin. of Δ^1-hydrocortisone and hydrocortisone.

pionate also are not eluted from Florisil in the 17-OHCS-containing 25% methanol-in-chloroform fraction(8).

Mean plasma 17-OHCS concentrations as a function of time following the oral administration of Δ^1-hydrocortisone and hydrocortisone are shown in Fig. 2. At a dose of 5 mg, Δ^1-hydrocortisone produced elevations in plasma 17-OHCS concentrations of about 70% of those obtained with 10 mg of hydrocortisone. At the 20 mg level, Δ^1-hydrocortisone gave plasma 17-OHCS levels which were higher at the maximum and which remained higher throughout the experimental period. At the 40 mg dose, the higher maximum values and prolonged elevations were even more striking. In addition, although 20 mg of Δ^1-hydrocortisone produced maximum plasma 17-OHCS concentrations of only about 60% of those observed after 40 mg of hydrocortisone, the response curves crossed after about 4 hours. This indicated a difference in the rates of removal of the 2 steroids from the circulation.

Plasma 17-OHCS half-life values obtained following the intravenous administration of Δ^1-hydrocortisone and hydrocortisone as the free steroids and as the sodium hemisuccinates are shown in Table I. Typical first order regression curves (method of least squares) are shown in Fig. 3. The mean half-lives of Δ^1-hydrocortisone and hydrocortisone were 71.3 and 52.1 minutes respectively. When administered as the sodium hemisuccinates, the mean plasma 17-OHCS half-lives were 79.7 and 67.0 minutes respectively. Therefore, Δ^1-hydrocortisone is removed from the circulation more slowly than hydrocortisone in the dog.

Discussion. The data presented indicate that orally administered Δ^1-hydrocortisone produced greater and more prolonged elevations of plasma 17-OHCS in the dog than did equivalent doses of hydrocortisone. The half-life of intravenously administered Δ^1-hydrocortisone was also longer than that of hydrocortisone (ratio 1.37). Further, a comparison of the ratios of plasma 17-OHCS concentrations one hour after oral administration shows the following: 10 mg (assuming linearity of

TABLE I. Half-Lives of Δ^1-Hydrocortisone and Hydrocortisone in the Dog.

	Half-life (min. ± stand. dev.)			
	Δ^1-Hydrocortisone		Hydrocortisone	
Dog	Alcohol	Hemisuccinate	Alcohol	Hemisuccinate
500	69.1 ± 2.1	89.8 ± 6.4	51.0 ± 2.2	59.7 ± 5.4
503	70.4 ± 2.0	—	45.1 ± .9	—
505	76.2 ± 3.9	72.8 ± 3.0	59.2 ± 1.4	87.3 ± 2.8
508	69.3 ± 1.6	—	53.1 ± 2.0	57.7 ± 2.2
502	—	76.4 ± 3.1	—	63.1 ± 2.2
Mean ± S.E.	71.3 ± 1.7	79.7 ± 5.2	52.1 ± 2.9	67.0 ± 6.9

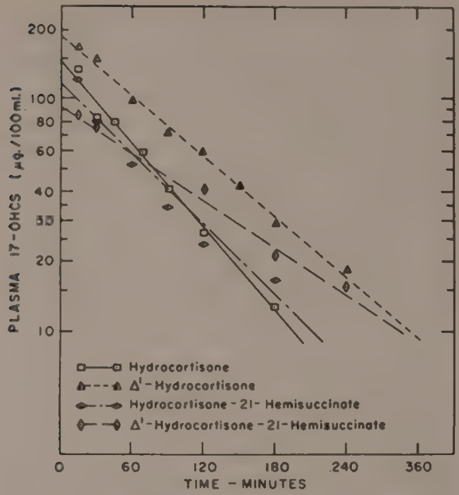

FIG. 3. Typical first order regression curves following intrav. admin. of Δ^1-hydrocortisone, hydrocortisone and the respective hemisuccinates.

response between 5 and 10 mg), 1.42; 20 mg, 1.28; and 40 mg, 1.73. Similarity of these ratios to the ratio of half-lives suggests that there is no appreciable difference in the rates of absorption of Δ^1-hydrocortisone and hydrocortisone from the gastrointestinal tract of the dog. It therefore appears that the higher and more prolonged elevations of plasma 17-OHCS following oral administration are a reflection of the reduced rate of metabolism of the Δ^1-steroid.

With the intravenously administered steroid hemisuccinates, the plasma 17-OHCS half-lives were greater than observed with the corresponding free alcohols (Table I). These values are not half-lives of the administered esters since only free 17-OHCS are measured by the analytical procedure. Apparently, hydrolysis of the hemisuccinates proceeds at a rapid but finite rate and the procedure determines the intermediate in a series of consecutive reactions such as: steroid hemisuccinate → 17-OHCS → metabolite(s). Jakoby and Tomkins(10) have studied the metabolism of pregnan-21-ol-3,20-dione-21-hemisuccinate by rat liver preparations and have reported that hydrolysis of the ester, followed by reduction of the C_3 carbonyl, analogous to the scheme above, represents the main metabolic pathway. However, they also observed considerable reduction of the C_3 carbonyl without prior hydrolysis. The Δ^1-hydrocortisone and hydrocortisone hemisuccinates probably behaved similarly. The somewhat larger standard deviations obtained (Table I) when the data are fitted as a first order regression (Fig. 3) indicate that the plasma 17-OHCS half-lives so obtained represent the simplification of a complex series of consecutive reactions. It may only be concluded that intravenous administration of the steroid esters results in somewhat lower but, due to continuing hydrolysis of the esters, more prolonged elevations of plasma 17-OHCS.

Metabolism of corticosteroids containing the Δ^4-3-ketone structure proceeds via reduction of first the C_4 unsaturation and then the C_3 carbonyl to produce tetrahydro derivatives which are subsequently conjugated(11). Alternatively, some reduction of the C_{20} carbonyl may also occur(12,13,14). The longer half-life of Δ^1-hydrocortisone may reflect the rate of an additional reduction at C_1 prior to the normal metabolism or may reflect a change in the relative proportions of the alternative processes. The recent studies by Liddle and Richard(15) and by Silber and Morgan(16) have shown that alteration of ring A or substitution of fluorine at C_9 of the hydrocortisone molecule results in a reduced rate of metabolism. These investigators have suggested, on the basis of urinary excretion studies, that changes in the steroid molecule alter its susceptibility to the usual enzymatic attack on ring A. Glenn* has observed that the 2-methyl-, 9α-fluoro-, and Δ^1-derivatives of hydrocortisone are metabolized at a markedly reduced rate, when compared with hydrocortisone, in a microsome-supernatant fraction of rat liver. Hence, it appears that enhanced potencies of these steroids are related to a reduced rate of metabolism in the animal. Final evaluation of the processes involved awaits further experimentation.

Summary. The effects of orally and intravenously administered Δ^1-hydrocortisone and hydrocortisone on plasma 17-OHCS concentrations in the dog have been studied. Δ^1-

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* Unpublished data.

Hydrocortisone produced higher and more prolonged plasma 17-OHCS elevations than did equivalent doses of hydrocortisone administered orally. The half-life of Δ^1-hydrocortisone was found to be 1.37 times that of hydrocortisone. Intravenous administration of these steroids as the hemisuccinates gave lower but somewhat more prolonged elevations in plasma 17-OHCS concentrations than the corresponding free alcohols. It was concluded that Δ^1-hydrocortisone was metabolized more slowly than hydrocortisone and suggested that this may explain, at least in part, the enhanced potency of the Δ^1-steroid.

1. Bunim, J. J., Pechet, M. M., and Bollett, A. J., *J. Am. Med. Assn.*, 1955, v157, 311.

2. Spies, T. D., Stone, R. E., Lopez, G. G., Tellechea, C. M. D., Toca, R. L., Reboredo, A., and Suarez, R. M., *ibid.*, 1955, v159, 645.

3. Spies, T. D., Stone, R. E., and Spies, H. A., Jr., *G. P.*, 1955, v12, 73.

4. Thorn, G. W., Renold, A. E., Morse, W. I., Goldfien, A., and Reddy, W. J., *Ann. Int. Med.*, 1955, v43, 979.

5. Ely, R. S., Done, A. K., and Kelley, V. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 503.

6. Nelson, D. H., and Samuels, L. T., *J. Clin. Endocrinol. and Metab.*, 1952, v12, 519.

7. Eik-Nes, K., Nelson, D. H., and Samuels, L. T., *ibid.*, 1953, v13, 1280.

8. Kelley, V. C., Done, A. K., Ainger, L. E., Ely, R. S., Forist, A. A., and Collins, E. J., *J. Pediatrics*, 1956, v49, 26.

9. Porter, C. C., and Silber, R. H., *J. Biol. Chem*, 1950, v185, 201.

10. Jakoby, W. B., and Tomkins, G., *Science*, 1956, v123, 940.

11. Tomkins, G., and Isselbacher, K. J., *J. Am. Chem. Soc.*, 1954, v76, 3100.

12. Glenn, E. M., and Recknagel, R. O., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 153.

13. Recknagel, R. O., and Glenn, E. M., *ibid.*, 1955, v89, 156.

14. Glenn, E. M., Siu, P. M. L., and Recknagel, R. O., *ibid.*, 1956, v91, 332.

15. Liddle, G. W., and Richard, J. E., *Science*, 1956, v123, 324.

16. Silber, R. H., and Morgan, E. R., *Clin. Chem.*, 1956, v2, 170.

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Titration of Influenza Virus and Viral Antibodies by Preserved Human Erythrocytes.* (22760)

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Procedures for titration of influenza viruses and their antibodies by use of chicken and human erythrocytes in tube hemagglutination (HA) and hemagglutination inhibition (HAI) tests have been well established(1-8). Most of the presently used tube HA and HAI titrations are read by the pattern method(5) and employ freshly washed chicken or human erythrocyte suspensions. Flick(9) reported the use of formalin treated human erythrocytes in studying the hemagglutinating capacity of influenza A virus; however, these cells exhibited spontaneous agglutination and could not be read by the pattern technic. Previous work in this laboratory(10) has demonstrated effective preservation of sheep erythrocytes for use in various serological systems. This report describes the preparation and use of preserved human erythrocytes in the titration of influenza viruses and their antibodies.

Materials and methods. Diluent used in all serological tests consisted of 0.137 M NaCl, 0.01 M Na_2HPO_4, and 0.003 M KH_2PO_4 in distilled water, the pH being 7.2 ± 0.05. Blood group O, Rh+ human erythrocytes were used in all tests. Those used as fresh cell suspensions were obtained by bleeding into an equal volume of Alsever's solution, and before use they were washed 3 times in 40 volumes of diluent and resuspended to a final concentration of 0.5%. Egg-adapted departmental strains of the type A (PR8 and IA43),

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type A′ (Benson and USD 2/53), and type B (Lee) influenza viruses were used throughout this study, representing pools of chorioallantoic and amniotic fluid viruses respectively. IA43 strain was originally obtained from Dr. A. P. McKee, University of Iowa, and the Benson strain from Dr. Jonas Salk, University of Pittsburgh. Virus preparations were stored at –20°C. Antiviral sera were prepared as pools from several immunized rabbits and stored at –20°C. Normal rabbit serum was prepared in a similar fashion from non-immunized rabbits. Portions of pre-immunization sera, as well as immune sera, were treated with receptor-destroying enzymes (RDE) prepared from filtrates of the 4Z strain of *Vibrio cholerae* according to the method suggested by the Expert Committee on Influenza(8). Following treatment with RDE all sera were inactivated by heat at 56°C for one hour. *Preparation of Preserved Human Erythrocytes (PHE).* PHE were prepared in a manner similar to that previously reported for preparation of sheep erythrocytes(10), with two major exceptions. Defibrinated group O, Rh+ human blood was always used, and erythrocytes were exposed to formalin twice. Forty ml of 50% formalin-diluent (equal volumes of formalin and twice concentrated diluent above) were rapidly mixed with 100 ml defibrinated human blood previously diluted with an equal volume of diluent. Formalinized blood was placed in a 37°C waterbath for 1½ hours with frequent mixing. Cells were then centrifuged and washed twice in at least 50 volumes of diluent each time, taking care to remove and discard by aspiration the top thin light colored coat after the first centrifugation. The sediment was reconstituted to 100 ml in diluent, again rapidly mixed with 40 ml of 50% formalin diluent, and held in the 37°C waterbath for another 1½ hour interval. The cells were then centrifuged and washed 6 times in at least 50 volumes of diluent each time. The washed and packed erythrocytes were then resuspended in diluent containing 0.3% formalin to approximately 10% by volume. Cell volume was then more accurately adjusted to 4% by centrifuging 5.0 ml of the 10% suspension in a Kolmer or vaccine tube and determining the volume of packed cells. As an additional precaution against contamination during use of the cells after preparation, 0.25 ml of a dye mixture (1 part of 1% aqueous crystal violet plus 2 parts of 1% aqueous brilliant green) was added and mixed with each 100 ml of 4% suspension. Addition of the dye also facilitated reading. PHE was dispensed in screw-cap bottles or dropper-vials and stored in the refrigerator. Tube HA and HAI titrations were carried out with 0.5% suspensions of both freshly washed human erythrocytes and PHE as described by the Expert Committee on Influenza(8), with the exception that HAI titers are expressed as reciprocals of final dilutions of sera. *Rapid Plate Titration of Influenza Virus.* CAF virus, undiluted and diluted 10^{-1} and 10^{-2}, was pipetted onto appropriate 2 cm rings on a glass plate, each virus dilution in volumes of .08, .04, .02, and .01 ml from left to right. Virus was pipetted with Bangs pipettes or 0.2 ml pipettes graduated in 0.01 ml divisions, and 1 drop (.025 ml) of PHE was immediately added to each ring. All 4 volumes of each virus dilution were mixed with PHE with a separate applicator stick from right to left, rotated for 1 to 2 minutes by hand and read macroscopically against a dark background over a strong oblique light in the usual manner of reading rapid plate bacterial agglutination tests. Each virus was run simultaneously with a diluent or CAF control. Undiluted virus volumes of .08, .04, .02, and .01 ml were found to correspond to virus dilutions of 1:100, 1:200, 1:400, and 1:800 respectively in the tube HA procedure; volumes of virus diluted 10^{-1} corresponded to tube HA virus dilutions 10 times those with undiluted virus, etc. HA titer of the virus was expressed as the highest corresponding dilution showing definite clumping, with negative diluent or CAF controls showing fine suspensions of cells, smoothly dispersed. Thus, if .02 ml of virus diluted 10^{-1} was the smallest amount showing definite clumping, the titer would be 1:4000. *Rapid Plate Titration of Virus Antibody.* All viral antisera were titrated against dilutions of CAF virus calculated to contain 5 HA units per .02 ml dose. Thus, if the virus titer were 1:2000 (2000 HA/ml), 5 HA would be contained in .02 ml undiluted virus; if virus titer were 1:8000, .02 ml virus diluted 1:4 would contain 5 HA. Inactivated serum, undiluted and diluted 10^{-1} and 10^{-2}, was pipetted onto appropriate rings on a glass plate, each serum dilution in volumes of .08, .04, .02, and .01 ml. Five HA units virus in .02 ml were then delivered to one side of each quantity of serum and 1 drop of PHE added. Reagents were mixed and read as indicated above. Undiluted serum volumes were found to correspond to tube HAI serum dilutions of 1:20, 1:40, 1:80, and 1:160 respectively, and decimal dilutions of serum corresponded to 10 fold tube HAI serum dilutions. Serum titer was expressed as highest corresponding dilution showing complete inhibition of agglutination, with diluent or negative serum controls showing marked agglutination.

Results. Preliminary observations on preparation of PHE paralleled those with preserved sheep erythrocytes(10). Two exposures to a final concentration of 14% formalin seemed optimal. Microscopically, in both stained and diluent preparations, the preserved erythrocytes stained well with some erythrocytes showing evidence of crenation. The addition of formalin diluent to whole blood rather than washed erythrocytes seemed advantageous; when washed erythrocytes were used, as reported by Flick(9), considerable lysis occurred. Furthermore, PHE showed considerable tendency to autoagglutinate when washed erythrocytes were exposed to final concentrations of formalin approaching 50%. PHE was found to be stable for several months, both from the standpoint of hemolysis and reactivity with influenza virus. Stable suspensions of PHE have been consistently prepared and maintained for a minimum of one year in the refrigerator. PHE suspensions were resistant to lysis in distilled water. A study of the optimal concentration of PHE for use in rapid plate procedures revealed that much less than a 4% suspension was too light to be easily read and too sensitive in the sense that the suspension seemed too granular. Much greater than 4% suspension was too heavy for easy reading and proved insufficiently sensitive with respect to the assigned virus dilutions when titrated by the rapid plate technic against known viruses. A 4% suspension was consistently found to yield end points in the rapid plate procedure in close agreement with tube hemagglutination titers, when virus dilutions were assigned to PHE-virus mixtures as described in above procedure for rapid plate titration of virus. PHE was used as 0.5% suspensions in all tube HA and HAI tests, in conformity with established procedures employing freshly washed human erythrocytes.

TABLE I. Comparison between PHE and Fresh Human Erythrocytes in Tube HA Titrations.

Virus	Virus pool	HA, units/ml Fresh RBC	PHE
PR8 (A)	1	2560	1280
	2	6400	6400
	3	5620	7680
IA43 (A)	1	2560	5120
	2	9600	12800
	3	15260	30520
IA43 (A) inact.*	2	3200	6400
Benson (A′)	1	1920	2560
	2	3200	6400
USD 2/53 (A′)		300	400
Lee (B)	1	120	120
	2	1600	2400
	3	1920	1280

* Inactivated at 56°C for 1 hr.

It is apparent (Table I) that PHE compared favorably with suspensions of freshly washed human erythrocytes in titrating various influenza viruses. In like manner, PHE and fresh human erythrocytes showed comparable activity in HAI titrations of antiviral sera produced in rabbits (Table II). The rapid plate technic employing PHE showed good correlation with tube procedures in both HA and HAI titrations (Tables III, IV).

TABLE II. Comparison between PHE and Fresh Human Erythrocytes in Tube HAI Titrations.

Antivirus*	RBC	Virus PR8	Benson	Lee
PR8 (A)	Fresh	10240†	1280	320
	PHE	20480	1280	320
Benson (A′)	Fresh	1280	5120	640
	PHE	640	5120	640
Lee (B)	Fresh	1280	640	10240
	PHE	640	1280	10240

* Antisera inactivated at 56°C for 30 min., but not RDE treated.

† Titers expressed as HAI units/ml.

TABLE III. Comparison between Tube and Rapid Plate HA Titrations Using PHE.

Virus	Virus pool	HA, units/ml Tube	HA, units/ml Rapid
PR8 (A)	5	2560	2000
	6	6400	4000
	3	7680	8000
Lee (B)	5	2560	2000
	6	6400	4000
	3	1280	1000
IA43 (A)	3	30520	20000
Benson (A′)	3	2560	2000

Discussion. Data in Table I illustrate typical findings in many tube HA titrations using both PHE and fresh human erythrocytes, discrepancies between which rarely have exceeded 1 full tube dilution. Good correlation exists when titrating A, A′, and B types of influenza virus, and it is interesting to note that PHE is equally susceptible to agglutination by influenza virus inactivated by heat. PHE shows equally good correlation with freshly washed human erythrocytes in both sensitivity and specificity in reciprocal HAI titrations of antiviral sera untreated (Table II) and treated with RDE (Table IV). Additional experiments involving tube and rapid plate HA and HAI titrations with both PHE and freshly washed human erythrocytes on serially diluted virus and antiviral sera have shown equally good correlation. PHE offers the distinct advantages of employing constant cellular antigens and eliminating the daily washing of erythrocytes in viral and antiviral titrations. In our experience the presently described rapid plate procedure using PHE provides an extremely rapid and sufficiently accurate method for titrating influenza virus and viral antibodies.

TABLE IV. Comparison between Tube and Rapid Plate HAI Titrations Using PHE.

Antivirus*	Titration	Virus PR8	Virus Lee
PR8 (A)	Tube	10240†	< 40
	Rapid	4000	<100
Lee (B)	Tube	< 40	5120
	Rapid	<100	4000
Normal rabbit serum	Tube	< 40	< 40
	Rapid	<100	<100

* All sera RDE treated and inactivated at 56°C for 1 hr.

† Titers expressed as HAI units/ml.

Summary. The preparation of PHE and their use in both tube and rapid plate HA and HAI procedures for the titration of influenza virus and viral antibodies are described. Such preserved cells have been stable, with respect to both resistance to lysis and sensitivity to hemagglutination by influenza virus, for a year. PHE showed good correlation in sensitivity with freshly washed human erythrocytes, and the rapid plate technic using PHE correlated well with tube HA and HAI procedures.

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1. Hirst, G. K., *Science*, 1941, v94, 22.
2. ———, *J. Exp. Med.*, 1942, v76, 195.
3. McClelland, L., and Hare, R., *Canad. J. Pub. Hlth.*, 1941, v32, 530.
4. Naval Laboratory Research Unit I, *U. S. Naval Med. Bull.*, 1943, v41, 114.
5. Salk, Jonas E., *J. Immunol.*, 1944, v49, 87.
6. Whitman, Loring, *U. S. Army Med. Bull.*, 1946, v6, 777.
7. ———, *J. Immunol.*, 1947, v56, 167.
8. Expert Committee on Influenza, World Hlth. Org. Rep. Ser. No. 64, 1953.
9. Flick, John A., PROC. SOC. EXP. BIOL. AND MED., 1948, v68, 448.
10. Cox, C. D., and Fingerhut, B., *J. Lab. and Clin. Med.*, 1956, v48, 298.

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Radioactive Rose Bengal Test as a Quantitative Measure of Liver Function. (22761)

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The radioactive rose bengal test, first described by Taplin, Meredith and Kade(1), promises to be one of the most sensitive known tests of liver function. By placing a radiation detector over the liver and injecting a small amount of I^{131}-tagged rose bengal intravenously, one can measure the rate of uptake and excretion of the dye. Normally, the activity rises to a peak in about 30 minutes and then falls as the dye is excreted via the biliary system into the bowel (Fig. 1). Where liver function is impaired, the rate of rise or fall, or both, is slower than normal. The original method for interpreting these uptake and excretion curves was semiquantitative, based on a visual comparison of each curve with a cluster of "normal" curves. Variations in size of patient, liver, or dose of tracer make this method quite unreliable. A purely quantitative analysis is described in the rest of this article.

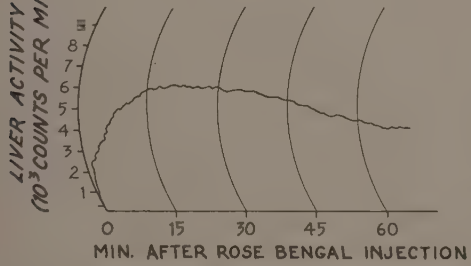

FIG. 1. A normal I^{131} rose bengal uptake excretion curve.

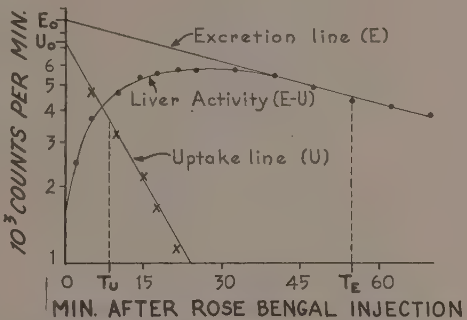

FIG. 2. Graphical analysis of the curve in Fig. 1. The uptake halftime (T_U) is 8 min., the excretion halftime (T_E) 55 min., and the liver blood volume $\left(\frac{E_o - U_o}{E_o}\right)$ is 18% of the total blood volume.

Method. It is apparent that, at any time, liver radioactivity (L) is equal to the difference between the amount of activity taken up $(1-U)$ and the amount excreted $(1-E)$, symbolically, $L = (1-U) - (1-E)$. Therefore, $L = E - U$. Both U and E decrease logarithmically with time; therefore, each of these components is characterized by a constant, which we may call "uptake halftime" and "excretion halftime," by analogy with the "halflives" of radioactive elements. The uptake halftime is the period in which the liver takes up half the circulating dye from the blood; the excretion halftime is the period in which half the amount taken up is excreted through the bile ducts.

If we consider as "time zero" the time after radioactive rose bengal has been injected into the bloodstream but before any has been taken up by liver cells, then, at this time: Liver radioactivity = radioactivity of blood in liver $= E_o - U_o$ and since E_o represents the total radioactivity injected,

$$R = \frac{\text{Liver Blood Volume}}{\text{Total Blood Volume}} = \frac{E_o - U_o}{E_o}.$$

The shape of each curve is determined by the 3 quantities, uptake halftime (T_U), excretion halftime (T_E), and fractional liver blood volume (R), and these constants can be found by graphical analysis.

Uptake excretion curves were obtained by the method of Taplin, Meredith and Kade(1) using a lead-shielded scintillation detector placed over the liver and aimed away from the gall bladder, with a counting rate meter

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* Fellow of National Foundation for Infantile Paralysis 1955-56.

TABLE I. Summary of Mean Values of Quantitative Radioactive Rose Bengal Test.*

Clinical diagnosis	No. of patients	Uptake halftime, min.	Excretion halftime, min.	Liver blood vol, % of total blood vol
Normal	35	9	92	18
Acute hepatitis	3	11	185	32
Recovered hepatitis	7	9	129	20
Laennec's cirrhosis	18	18	300	32
Biliary cirrhosis	3	28	2800	35
Heart failure	2	21	106	24
Acute ulcerative colitis, with liver damage	1	7	260	23
Same case, after improvement of colitis		6	145	18
Common bile duct obstruction (carcinoma of head of pancreas)	1	15	2200	23
Same case, 1 wk after surgery (Whipple procedure)		9	140	21

* These values were derived from 42 uptake excretion curves published by Taplin, Meredith and Kade, and 28 curves recorded at Stanford Hospital and the Palo Alto Clinic. Comparison of the two groups reveals very nearly the same avg values for patients with the same diagnosis. The stand. dev. of each value is close to ± 25%.

and recorder. Each patient was given intravenously about 25 μg of rose bengal in 1 cc of saline, having activity of about 25 μc. Each record was continued until the peak activity was passed and the counting rate was well on the down slope (Fig. 1). Each curve is analyzed as follows. After background activity is subtracted, the curve is transposed to semilogarithmic paper (Fig. 2). A straight line (E) is drawn from the ordinate through the descending limb; this is the excretion line. The uptake line (U) is obtained by plotting differences between E and the rising portion of the curve, and drawing another straight line from the ordinate through these points. The uptake halftime is the time when $U = \frac{1}{2} U_o$, the excretion halftime is the time when $E = \frac{1}{2} E_o$. Fractional liver blood volume, $R = \frac{E_o - U_o}{E_o} \times 100\%$.

Results. Average values derived from data in this clinic and from the published curves of Taplin, Merédith and Kade(1) are presented in Table I. The standard deviation is about ± 25% in each group. The cases of ulcerative colitis and carcinoma of the head of the pancreas show how the test results in single individuals change with altered clinical state.

Discussion. From this series, it appears that hepatocellular damage is manifested by delayed uptake and excretion, obstructive processes cause delayed excretion, and circulatory failure, as expected, reduces the uptake rate. This test appears to have great practical value in the differential diagnosis of jaundice and liver disease.

Summary. The radioactive rose bengal test, a very sensitive test of liver function, can be analyzed graphically to yield three quantities —uptake halftime, excretion halftime and fractional liver blood volume. The values in various liver diseases seem to be characteristic of the disease and to indicate whether cellular damage or obstruction predominates.

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1. Taplin, G. V., Meredith, D. M., Jr., and Kade, H., *J. Lab. and Clin. Med.*, 1955, v45, 665.

Received September 5, 1956. P.S.E.B.M., 1956, v93.

Postictal Recovery in Normal and Diphenylhydantoin (Dilantin)-Treated Mice.* (22762)

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It is well known that supramaximal electroshock stimulation of experimental animals evokes seizures characterized by 3 components: tonic hindleg flexion, tonic hindleg extension, and terminal generalized clonus. Alteration in the severity of this seizure, by drugs or experimental procedures, may be recognized by changes in duration of the tonic components of the seizure. It was shown(1) in mice that relatively *minor* changes in seizure severity can be detected by changes in duration of tonic flexion, whereas *major* changes in seizure severity can be recognized by changes in duration of both the flexor and the extensor tonic components. These criteria for changes in seizure severity have now been employed to investigate the postictal recovery process in normal and diphenylhydantoin (Dilantin)-treated mice. The results of this investigation provide the basis for this report.

Methods. A population sample of 160 adult male albino mice obtained from the Carworth Farms (CF #1 strain) was randomly divided into 2 groups (test and control) of 80 mice each. Each animal of the test group was given orally 11.0 mg/kg of diphenylhydantoin sodium (Dilantin Sodium) suspended in 10% acacia mucilage, *i.e.*, approximately an ED_5 as previously determined by the maximal electroshock seizure (MES) test; the control group was given an equivalent volume of 10% acacia mucilage. Three hours later, at the time of peak anticonvulsant effect of Dilantin by the MES test, groups of 8-10 mice (test and control) were given an initial electroshock of 50 mA (0.2-sec. duration, 60-cycle a.c., corneal electrodes) and the pattern of the "first" seizure was recorded. At various time intervals after the initial shock, a second electroshock of the same intensity was administered and the pattern of the "second" seizure was recorded. Any one animal, however, received only 2 electroshocks. The time interval between electroshocks varied from 180 to 540 seconds for Dilantin-treated mice and from 40 to 200 seconds for control mice. The mean durations of hindleg tonic flexion and extension, terminal generalized clonus, and total seizure for both "first" and "second" convulsions were calculated as follows: the durations of the individual components were summed and the values obtained divided by the total number of mice per group. In addition, the standard deviations of the means were calculated. The endpoints for these seizure components have been described previously(1). The time (RT_{50}) required for 50% of mice to recover the ability to exhibit a second tonic-clonic seizure, regardless of duration of components, after a first maximal convulsion was determined by the graphic log-probit method of Litchfield and Wilcoxon(2).

Results. The mean durations of the various components of "first" seizures and of "second" seizures elicited at the indicated times in normal mice are shown in Table I. It may be seen from the table that the mean durations of the various components and the total duration of "first" seizures were remarkably uniform, and there was no significant difference in the values obtained in the various groups of normal mice. As might be expected, the character and the duration of the "second" seizure are crucially dependent on the time, in the postictal period, of application of the second electroshock. The longer this time is extended, the more the "second" resembles the "first" seizure. Whereas, at 200 seconds, the mean tonic flexion duration of the "second" seizure was still significantly

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TABLE I. Mean Durations of "First" and "Second" Seizure Components in Normal Mice.

Time of application of 2nd electroshock (sec.)	Mean duration of seizure components, seconds[†]							
	Tonic hindleg							
	Flexion		Extension		Clonus		Total duration	
	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure
40	1.6 ± .1	6.9 ± .4	13.8 ± .7	0	6.4 ± .7	8.6 ± .7	21.8 ± .7	15.5 ± .9
60	1.6 ± .4	8.2 ± .7	13.8 ± .4	1.4 ± .9	6.4 ± .6	11.0 ± .6	21.9 ± .6	20.6 ± .6[*]
80	1.5 ± .2	5.9 ± 1.1	14.0 ± .5	4.9 ± 1.3	7.0 ± .6	8.4 ± .6[*]	22.5 ± .9	19.2 ± 1.7[*]
100	1.8 ± .2	5.0 ± .9	14.1 ± .3	7.2 ± 1.8	6.7 ± .3	7.9 ± .7[*]	22.5 ± .7	20.1 ± 1.0[*]
120	1.4 ± .1	2.7 ± .3	15.7 ± .7	12.6 ± .8	7.8 ± .3	9.1 ± .8[*]	24.9 ± .8	24.4 ± 1.2[*]
140	1.6 ± .1	2.8 ± .2	14.2 ± .4	11.1 ± .9	7.4 ± .2	7.5 ± .8[*]	23.1 ± .5	21.4 ± .9[*]
160	1.6 ± .1	2.6 ± .2	14.1 ± .5	13.5 ± 1.0[*]	7.0 ± .8	8.6 ± 1.0[*]	22.7 ± 1.0	23.6 ± 1.5[*]
180	1.6 ± .1	2.2 ± .2	14.1 ± .8	14.6 ± .9[*]	7.9 ± .8	8.8 ± .9[*]	22.8 ± .5	24.7 ± 1.1[*]
200	1.5 ± .1	2.3 ± .1	14.7 ± .6	14.1 ± 1.1[*]	7.3 ± .7	7.2 ± .8[*]	23.5 ± .7	23.6 ± .9[*]

[*] Not significantly different from mean value of the first seizure (P >.05 .

[†] Sum of the durations of individual components divided by total No. of mice per group ± standard deviation of the mean.

longer than that of the "first" seizure, the mean durations of tonic extension, terminal clonus and total convulsion of "second" seizures were not significantly different from those of the control values after 160, 80, and 60 seconds, respectively. Thus, the order in which the durations of the various components of the "second" seizure returned to control values was as follows: total seizure duration, terminal clonus, tonic extension, and tonic flexion.

The mean durations of the various components of "first" and "second" seizures elicited at the indicated times in Dilantin-treated mice are shown in Table II. As shown in the table, there was no significant difference in the "first" seizure values obtained in groups of Dilantin-treated mice. The mean durations of tonic flexion, tonic extension, terminal clonus, and total convulsion of "second" seizures were not significantly different from their appropriate control values after 540, 400, 320, and 180 seconds, respectively. Thus, the order in which the durations of the various components of the "second" seizure returned to control values was the same as observed for untreated mice, *i.e.*, total seizure duration, terminal clonus, tonic extension, and tonic flexion.

The RT_{50} and 95% confidence limits for control mice were 75 (65-86) seconds, whereas those for Dilantin-treated animals were 320 (260-394) seconds. The Dilantin-induced increase in RT_{50} was highly significant ($P < .001$), and amounted to more than 300%.

Discussion. The data presented indicate that the pattern of a "second" maximal seizure elicited after a "first" depends on the time interval between the two seizures and

TABLE II. Mean Durations of "First" and "Second" Seizure Components in Dilantin Treated Mice.

Time of application of 2nd electroshock (sec.)	Mean duration of seizure components, seconds[†]							
	Tonic hindleg							
	Flexion		Extension		Clonus		Total duration	
	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure	1st seizure	2nd seizure
180	2.8 ± .2	7.8 ± .8	9.6 ± .7	1.3 ± .7	8.8 ± .8	11.3 ± 1.1	21.0 ± .9	22.8 ± .9[*]
280	2.9 ± .1	6.8 ± 1.0	9.2 ± .9	2.6 ± 1.3	7.8 ± .8	12.3 ± 1.0	19.7 ± 1.4	22.8 ± .9[*]
320	3.1 ± .3	6.3 ± 1.0	8.7 ± .8	4.8 ± 1.5	8.6 ± .6	9.0 ± .3[*]	20.5 ± .4	20.1 ± .9[*]
360	2.4 ± .2	6.0 ± 1.1	12.7 ± .8	6.9 ± 1.9	8.5 ± .9	11.1 ± 1.4[*]	23.6 ± 1.0	24.0 ± 1.0[*]
400	2.9 ± .3	5.4 ± .8	10.9 ± 1.0	7.2 ± 1.6[*]	7.5 ± .9	10.0 ± .7[*]	21.5 ± .6	22.6 ± .7[*]
480	3.2 ± .5	5.3 ± .8	10.2 ± .9	6.9 ± 1.6[*]	9.2 ± .7	11.6 ± 1.5[*]	22.3 ± .7	23.7 ± 1.2[*]
540	2.4 ± .3	3.5 ± .5[*]	12.0 ± 1.2	12.3 ± 1.4[*]	9.5 ± .6	8.9 ± .8[*]	24.0 ± .9	24.7 ± 1.1[*]

[*] Not significantly different from mean value of the first seizure (P >.05).

[†] Sum of the durations of individual components divided by total No. of mice per group ± standard deviation of the mean.

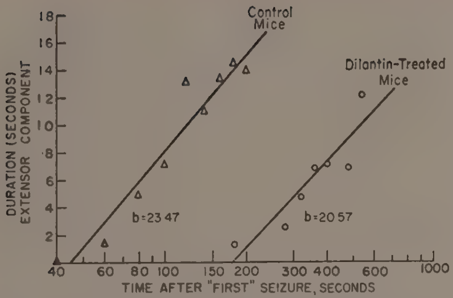

FIG. 1. Rate of recovery of hindleg tonic-extensor component in control and Dilantin-treated mice. Time interval in sec. (logarithmic scale) between "first" and "second" seizures is shown on abscissa and duration of extensor component in sec. (linear scale) is shown on ordinate. Values obtained in control mice are plotted with triangles; those obtained in Dilantin-treated mice, with circles. The continuous lines were fitted to the plotted points by the method of least square. The letter b indicates the calculated slope of the line. See text for discussion.

that the total duration of a maximal electroshock seizure is the sum of the durations of the individual seizure components. Dilantin prolonged by more than 170% the time necessary for "second" seizures to return to "first" seizure values and induced a 325% increase in RT_{50}. Previous studies from this laboratory indicate that Dilantin also increases RT_{50} in rats(3).

By what mechanism does Dilantin prolong recovery time? Interesting information on this question is revealed by plotting the duration of the hindleg tonic extensor component of "second" seizures elicited in normal and Dilantin-treated mice against the time interval between seizures. This plot is shown in Fig. 1 from which it can be seen that recovery time, as measured in these experiments, is composed of two factors: the recovery time *per se* (indicated by the slopes of the two regression lines) and the latent period before recovery of the tonic-extensor component starts (indicated by the points of intercept of the regression lines and abscissa). Since there is no significant difference in the slopes of the recovery regression lines for control and Dilantin-treated mice and since the time before recovery starts is 45 seconds in control mice and 175 seconds in Dilantin-treated mice, it would appear that the *rate* of recovery of tonic extensor ability is unaffected by this drug and that it acts primarily to prolong the latent period before recovery of the tonic-extensor component starts. This interpretation is supported by the work of Esplin who demonstrated[‡] on cat spinal cord preparations that Dilantin had little effect on the level of resting excitability, as judged by transmission of an isolated impulse, but deepens the depression following an impulse.

In recent years, we have directed considerable attention to the effects of various physiological vicissitudes, drugs, etc. on the RT_{50} for maximal electroshock seizures(1,3, 4,5); however, the data presented in Tables I and II represent our first precise measurement of the time for recovery of "second" maximal seizures as related to "first" seizure values. What percentage of CNS reactivity has been regained at the time that 50% of animals again show some detectable tonic-extensor component? The return of CNS reactivity is logarithmic; hence, a definitive answer to this question can not be derived from the data presented. Nevertheless, an interesting correlation can be revealed by comparing recovery of the tonic-extensor component and RT_{50}. It may be determined from the tables that the mean durations of the tonic-extensor component of "first" seizures are 14.27 and 10.47 seconds in normal and Dilantin-treated mice, respectively. By reference to Fig. 1, it may be seen that the times for recovery of one-half the full tonic-extensor durations are 93 and 320 seconds in normal and Dilantin-treated mice, respectively. Since the respective RT_{50}s were determined to be 75 (65-86) and 320 (260-394) seconds, it may be concluded that RT_{50} is approximately the time for recovery of 50% of the *full* tonic-extensor component. Because of the comparative ease of its determination and calculation, the RT_{50} is a more useful and accurate endpoint for the measurement of recovery of CNS reactivity than is full recovery of the tonic-extensor component.

Summary. In order to study postictal recovery, groups of normal and Dilantin-treated mice were given a supramaximal electroshock and the durations of the hindleg components of the "first" seizure recorded. At various time intervals after the "first" seizure, a "second" supramaximal electroshock was given and the durations of the hindleg components of the "second" seizure recorded. This procedure was repeated, with longer time intervals between seizures, until the observed durations of the "second" seizure components were not significantly different from their "first" seizure values. In addition, the time (RT_{50}) required for 50% of animals to recover ability to exhibit a second tonic-clonic seizure after a first was determined. The data obtained may be summarized as follows: The order in which the durations of the various components and the total duration of the "second" seizure returned to their "first" seizure values were the same in both groups: total seizure duration, terminal clonus, tonic extension, and tonic flexion. The total duration of a maximal seizure is relatively independent of marked changes in duration of the individual seizure components. Dilantin prolonged the recovery time of terminal clonus, tonic extension, and tonic flexion by approximately 300%, 150%, and 170%, respectively. In addition, Dilantin prolonged RT_{50} by 325%. Evidence is presented which suggests that Dilantin acts to prolong the latent period before recovery of the tonic-extensor component starts, and that the recovery process *per se*, once initiated, is relatively unaffected by the drug.

[‡] *Fed. Proc.*, 1955, v14.

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1. Tedeschi, D. H., Swinyard, E. A., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1956, v116, 107.

2. Litchfield, J. T., Jr., and Wilcoxon, F., *ibid.*, 1949, v96, 99.

3. Swinyard, E. A., Tedeschi, D. H., and Goodman, L. S., *Fed. Proc.*, 1953, v12, 370.

4. Swinyard, E. A., and Toman, J. E. P., *Am. J. Physiol.*, 1948, v154, 207.

5. Woodbury, D. M., Hurley, R. E., Lewis, N. G., McArthur, M. W., Copeland, W. W., Kirschvink, J. F., and Goodman, L. S., *J. Pharmacol. and Exp. Therap.*, 1952, v106, 331.

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Dehydrogenase Activity of Rat Epiphyseal Cartilage.* (22763)

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Some years ago dehydrogenase activity was demonstrated in epiphyseal cartilage, using succinate and citrate as substrates and the decolorization of methylene blue as an indication of enzyme action(1). This technic does not allow for any precise localization of the enzymes in the cell. Hence, the application of newer technics utilizing tetrazolium compounds was undertaken, since so little is known about the metabolic activities of growing cartilage.

Materials and methods. Young (40-60 g) rats were killed by a blow on the head. After the lower extremity was dissected of muscle, free hand sections, as thin as possible, were made with a razor through the epiphyseal cartilages of the upper tibia or lower femur. Such slices were washed briefly in saline to remove any blood and were placed in depression slides containing 4 drops of .2M phosphate

TABLE I. Degree of Tetrazolium Reduction in One Hour with Different Substrates.

Control	0
Succinate	+++
Succinate and malonate	0
Citrate	++
Isocitrate	+++
Lactate	+++
Malate	+++
Pyruvate	0
Glucose	0
Glutamate	0
Oxalacetate	+
Xanthine	0

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* Aided in part by grant from American Cancer Society, upon recommendation of Committee on Growth.

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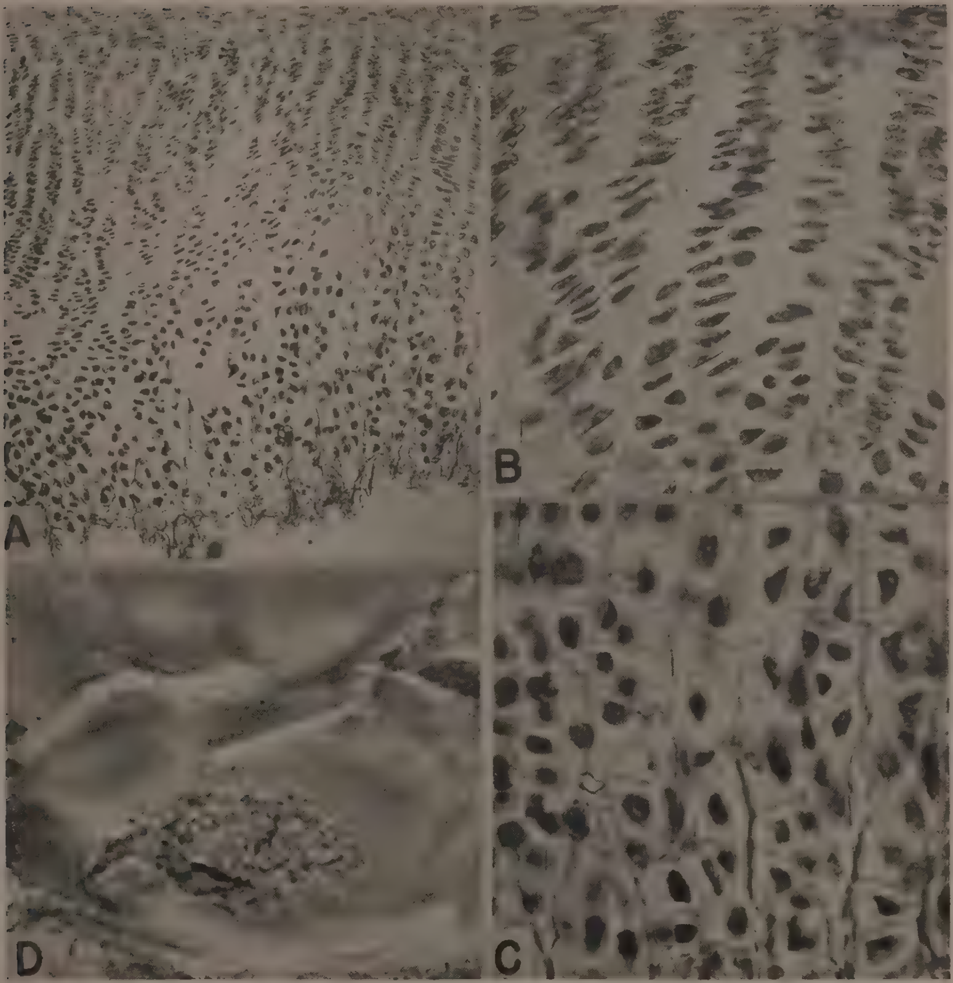

FIG. 1. Microphotographs of rat epiphyseal cartilage slices incubated with buffered sodium succinate and neotetrazolium chloride. A. Entire width of epiphyseal cartilage. ×75 (A.F.I.P. neg. 56-5005). B. Higher power of zone of proliferating cartilage cells. ×260 (A.F.I.P. neg. 56-5006). C. Higher power of zone of hypertrophic cells. ×260 (A.F.I.P. neg. 56-5007). D. High power of single cartilage cell to show rods and granules of formazan about non-staining nucleus. ×1400 (A.F.I.P. neg. 56-15407).

buffer of pH 7.4, 2 drops of .2M substrate in phosphate buffer and 3 drops of neotetrazolium chloride (0.1%) in phosphate buffer. Coverslips were applied, care being taken to exclude air; the preparations were incubated at 37°C. The following substrates were employed; glucose, succinate, malonate, malate, isocitrate, oxalactate, pyruvate, lactate, glutamate, xanthine, alpha-ketoglutarate and alanine. Control slices were incubated with 2 drops of buffer substituting for substrate. The preparations were observed under the microscope every half hour, generally for at least 2 hours.

Results. The distribution of enzyme activity with succinate as a substrate will first be described. Purplish granules of formazan pigment began to appear after one half hour of incubation, reaching a maximum in 2 hours. Under the low power of the microscope such pigment deposits seemed to delineate the nuclei of the cartilage cells. Under higher power, however, these structures were seen to remain unstained; the granules and short rods of pigment were grouped around an unstained area (the nucleus) in the center of the cell. A wide clear zone was present between this pigment and cell membrane. Formazan pigment was found in the cartilage cells at all stages of development. During the time of observation no formazan pigment developed in the slices incubated with malonate or without substrate.

Variations in degree of intensity of the strain depending on the substrate used are shown in Table I. Those leading to the most pigment formation in the shortest period of time were succinate, isocitrate, citrate, lactate, and malate.

Discussion. These histochemical observations confirm the presence of dehydrogenase activity of epiphyseal cartilage described earlier(1) and amplify the few biochemical studies which have been carried out on articular(2) and epiphyseal(3,4) cartilages.

The epiphyseal and costal cartilages are unique in that they display all stages of differentiation and growth at a time when other tissues in the organism have matured. The needs of the epiphyseal cartilages for energy to grow must be great when it is realized that a rat's lower femur increases .18 mm in length per day(5). Hence, it is not surprising to find evidence for the active metabolism of certain compounds involved in the Krebs cycle. Exactly how such data may fit into the total metabolism of cartilage remains to be elucidated. These observations would appear to have no bearing on the calcification mechanism since activity was found in all strata of the epiphyseal cartilage. However, they provide some understanding for the presence of citric acid in cartilage(6) though they do not indicate what proportion may be utilized in the citric acid cycle and what amounts may be purely fortuitous, as in bone(7).

Summary. Utilizing the tetrazolium technic, intracellular dehydrogenase activity has been localized in epiphyseal cartilage cells. The most favorabe substrates appear to be succinate, isocitrate, citrate, lactate and malate.

1. Follis, R. H., Jr., and Berthrong, M., *Bull. Johns Hopkins Hosp.*, 1949, v85, 281.

2. Rosenthal, O., Bowie, M. A., and Wagoner, G., *J. Cell. and Comp. Physiol.*, 1942, v19, 15.

3. Dixon, T. F., and Perkins, H. R., *Biochem. J.*, 1952, v52, 260.

4. Hiatt, H. H., and Shorr, E., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 723.

5. Sissons, H. A., *J. Anat.*, 1953, v87, 228.

6. Dickens, F., *Biochem. J.*, 1941, v35, 1011.

7. Armstrong, W. D., and Singer, L., *Bone Structure and Metabolism.* Ciba Foundation Symposium, G. E. W. Wolstenholme, Editor, London, 1956, p103.

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Effect of Aminonucleoside on Ribonucleic Acid Content of Mouse Mammary Adenocarcinoma *in vivo.* (22764)

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Puromycin (6-dimethylamino-9-[3′-deoxy-3′(p-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl]purine), an antibiotic isolated by Porter *et al.*(1), and structurally characterized by Waller *et al.*(2) has been shown by Troy *et al.*(3) to inhibit the growth of certain experimental tumors. It has further been shown by Oleson *et al.*(4) that the carcinostatic activity for mouse mammary adenocarcinoma resides in the aminonucleoside portion of puromycin. The structure of the aminonucleoside suggests that its antitumor activity could involve nucleic acid metabolism. To test this hypothesis, C3H mammary adenocarcinomas were analyzed for RNA content and composition, and compared to the same tumor

TABLE I. Purine-Bound Ribose Content of C3H Mammary Adenocarcinomas.

Group	Treatment	Mean tumor wt, mg*	μM PBR per g tissue†
1	0.2 ml 0.9% NaCl intraper. twice daily for 2½ days	143	5.0
2		352	6.2
3		675	6.8
4		1800	6.3
5		2500	5.9
	Mean	1094	5.84‡
6	3 mg 2484L in 0.9% NaCl intraper. twice per day for 2½ days	220	2.8
7		362	3.5
8		625	3.8
9		1400	4.7
10		2320	4.0
	Mean	985	3.76‡

* Mean wt of similar sized tumors from 2 to 15 backcrossed C3H mice depending on tumor size.

† Micromoles of purine-bound ribose/g wet tissue.

‡ Difference between means of purine-bound ribose of the 2 treatments is statistically significant since P <0.008 by analysis of variance.

from mice which had been treated with 6-dimethylamino-9- [3′-deoxy-3′-amino-β-D-ribofuranosyl]purine (2484L).

Methods. In order that an appropriate comparison might be made, the established tumors on backcrossed C3H mice were so selected that the control and 2484L treated tumors would be of approximately equal size at the end of the treatment period. Mice bearing much larger tumors were selected for 2484L treatment than those intended for no treatment. The aminonucleoside (2484L), pre pared by the method of Baker *et al.*(5) was injected intraperitoneally into the mice in five 1.5 mg doses in 0.9% NaCl at intervals of about 12 hours. At the end of the treatment period, the mice were decapitated and the tumors rapidly excised and frozen in dry ice. The frozen tumors were homogenized in cold 6% $HClO_4$ and the ribonucleic acid extracted by a modified Schmidt-Thannhauser-Schneider procedure(6). The RNA was estimated as purine-bound ribose by the orcinol reaction (7). A portion of the ribonucleotide containing extract was paper chromatographed and the component monoribonucleotides estimated by the method of Elson *et al.*(8).

Results. The RNA (purine-bound ribose) content of 2484L treated tumors was significantly less than that of untreated controls

TABLE II. Ribonucleotide Distribution in C3H Mammary Adenocarcinomas.

Group*	Treatment	Mean tumor wt, mg	Adenylic acid		Cytidylic acid		Guanylic acid		Uridylic acid		Total μM mononucleotide/g	Ratio, μM purine to μM pyrimidine
			μM/g†	Base ratio‡	μM/g†	Base ratio‡	μM/g†	Base ratio‡	μM/g†	Base ratio‡		
1	.9% NaCl	143	2.30	1.00	3.33	1.45	4.00	1.74	1.59	.69	11.11	1.29
4		1800	2.63	1.00	3.92	1.50	4.34	1.65	2.36	.90	13.25	1.11
6	3 mg 2484L	220	1.20	1.00	1.64	1.37	2.08	1.73	.79	.66	5.71	1.36
9		1400	1.92	1.00	2.84	1.48	3.40	1.77	1.72	.90	9.90	1.17

* Group No. corresponds to tumor group in Table I.

† Micromoles/g wet tissue. All values are means of 3 determinations.

‡ Ratio of μM of base to that of adenine.

(Table I). The nucleotide composition of the RNA fraction of the tumors revealed no gross difference in base ratios (Table II). A comparison of the total ribonucleotide contents of these groups confirms the lower RNA concentration of the 2484L treated tumors. The effect of 2484L treatment on tumor RNA cannot be ascribed to hydration, since it was found that the total nitrogen per gram wet weight of similarly treated and control groups did not differ significantly. These results suggest that 2484L causes an inhibition of synthesis (or an acceleration of degradation) of RNA in C3H mammary carcinoma. Ledoux and Revell(9) have shown that the decrease in growth rate of an ascites tumor (Landschütz) is associated with a parallel decrease in RNA content under normal conditions of growth. Thus the inhibition of growth of this tumor by 2484L may be associated with the decrease in RNA content.

Summary. (1) The RNA content of 2484L treated C3H mammary adinocarcinomas was shown to be lower than that of untreated controls. (2) The nucleotide composition of both treated and control tumors appeared to be similar.

1. Porter, J. N., Hewitt, R. I., Hesseltine, C. W., Krupka, G., Lowery, J. A., Wallace, W. S., Bohonos, N., and Williams, J. H., *Antibiot. and Chemother.*, 1952, v2, 409.

2. Waller, C. W., Fryth, P. W., Hutchings, B. L., and Williams, J. H., *J. Am. Chem. Soc.*, 1953, v75, 2025.

3. Troy, W., Smith, S., Personeus, G., Moser, L., James, E., Sparks, S. J., Stevens, M., Halliday, S., Mackenzie, D., and Oleson, J. J., *Antibiotics Annual*, 1953, 186.

4. Oleson, J. J., Bennett, P. L., Halliday, S. L., and Williams, J. H., *Acta Union Internationale Contre Le Cancer*, 1955, v11, 161.

5. Baker, B. R., Joseph, J. P., and Williams, J. H., *J. Am. Chem. Soc.*, 1954, v76, 2838.

6. Volkin, E., and Cohn, W. E., *Methods of Biochemical Analysis*, D. Glick, (Editor), New York, Interscience Publishers, Inc., 1954, v1, 290.

7. ———, *ibid.*, 1954, v1, 298.

8. Elson, D., Gustafson, T., and Chargaff, E., *J. Biol. Chem.*, 1954, v209, 285.

9. Ledoux, L., and Revell, S. A., *Biochim. et Biophys., Acta*, 1955, v18, 416.

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Anaerobic Glycolysis of Brain Cortex Slices in Hyperthyroid and Control Rats.* (22765)

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The primary site of action of the thyroid hormone in the intermediary metabolic scheme cannot yet be considered as definitely established. An early theory first enunciated by Haffner shortly after the advent of the Warburg metabolic techniques, was that the thyroid hormone primarily affects a step involved in anaerobic glycolysis(1). This was disputed by McEachern who claimed that anaerobic glycolysis was not increased in the livers of rats made hyperthyroid, altho slices of such livers invariably showed increased oxygen consumption when compared with normal control liver-slice values(2). His data confirmed similar earlier work of Dresel(3). Furthermore Anselmino *et al.* had early claimed that spleen slices from hyperthyroid rats failed to show a rise in either oxygen consumption or anaerobic glycolysis in a comparison with normal controls(4). However, in view of a recent statement by Goldstein that anaerobic glycolysis is increased in hyperthyroid rats(5), it was decided to reinvestigate the problem of the relationship of thyroxin to glycolysis beginning with the anaerobic type and brain cortex slices for a reason to be discussed later.

Methods. L-thyroxine is dissolved in a

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TABLE I. Anaerobic Glycolysis of Brain Slices from Control and Hyperthyroid Rats.

Type of animal	No. of animals	No. of values	Anaerobic glycolysis expressed as $Q^{N_2}_{CO_2}$ *
Normal control rats	12	24	18.9 ± 2.34†
Hyperthyroid "	8	24	16.7 ± 3.66

* See text.

† Stand. dev. of mean.

minimal amount of 0.05N NaOH (1 mg in 0.1 cc of 0.05N NaOH). Two mg of such a thyroxine solution are injected subcutaneously into white Wistar rats weighing 100-200 g and this is followed by similar injections of 1 mg daily for 2-4 days, the thyroxine solution being made up fresh daily. The treated rats and untreated litter mates were fed *ad lib* with Purina rat checkers and water. All rats were decapitated for the experimental runs and cerebral cortex slices prepared from each hemisphere manually with a double-edged razor blade held by a Kelly Clamp. One or two slices, less than 0.5 mm thick, were placed into Warburg flasks each containing 3 cc of Krebs-Ringer-Bicarbonate-0.1% glucose solution(6). The vessels were gassed for 5 minutes with a 5% CO_2-95% N_2 gas mixture and placed in a Warburg bath at 37°C. After an 8-minute temperature equilibration period, CO_2 release from the bicarbonate buffer due to anaerobic glycolysis was measured every 15 minutes for 1 hour.

Results. The results are expressed in Table I as cmm CO_2 released per mg dry weight of brain cortex per hour $(Q^{N_2}_{CO_2})$, the slices having been dried for 24 hours at 100°C.

Discussion. Since anaerobic glycolysis in the liver is extremely low and possibly even rudimentary, the fact that it does not increase in liver slices(2-4) from hyperthyroid animals might be considered of doubtful significance. Brain cortex slices, like spleen, however, could be of value as a test for a primary thyroid hormone effect on glycolysis since they show no rise in oxygen consumption in the hyperthyroid state, but do possess a high rate of anaerobic glycolysis normally. Thus a rise in anaerobic glycolysis in hyperthyroid brain slices could not be considered as secondary to a rise in O_2 consumption, since the latter does not occur(7). The fact that such brain slices from hyperthyroid rats do *not* exhibit a higher rate of anaerobic glycolysis than similar slices from normal rats corroborates similar findings with spleen slices reported by Anselmino *et al.*(4). These results combined with those of Dresel(3) and McEachern(4) make the hypothesis of a primary effect of the thyroid hormone on anaerobic glycolysis very unlikely, considering especially that positive, physiological effects on the brain in different thyroid states have been reported(8) and are known clinically. The possibility must still be considered, however, that all the positive brain findings are secondary and due to influence of the thyroid gland upon other glands of internal secretion. If such were the case, then it is possible that the thyroid hormone has no direct effect on any phase of brain metabolism.

Conclusion. Anaerobic glycolysis is *not* increased in brain slices from rats injected with 4-8 mg of L-thyroxine, when compared to similar values from normal control rats.

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1. Haffner, F., *Klin. Wchnschr.*, 1927, v6, 1932.

2. McEachern, D., *Bull. Johns Hopkins Hosp.*, 1935, v56, 145.

3. Dresel, K., *Klin. Wchnschr.*, 1928, v7, 504.

4. Anselmino, K. J., Eichler, O., and Schlossimann, H., *Biochem. Z.*, 1929, v205, 481.

5. Goldstein, M. S., *J. Biol. Chem.*, 1952, v199, 923.

6. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques*, Burgess Publishing Co., Minneapolis, Minn. 1st Ed., p194.

7. Spirtes, M. A., PROC. SOC. EXP. BIOL. AND MED., 1941, v46, 279.

8. Woodbury, D. M., Hurley, R. E., Lewis, N. G., MacArthur, M. W., Copeland, W. W., Kirschvink, J. F., and Goodman, L. S., *J. Pharmacol. Exp. Therap.*, 1952, v106, 331.

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Development of Resistance and Cross-Resistance *in vitro* to Erythromycin, Carbomycin, Spiramycin, Oleandomycin and Streptogramin.* (22766)

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Among the major problems in the field of infectious diseases today, particularly in hospitals, is the occurrence, with increasing frequency, of strains of *Micrococcus pyogenes,* var. *aureus,* resistant to many antibiotics that are most commonly used. This has been accompanied by a serious increase in incidence and severity of infections due to such organisms and in difficulties encountered with the use of antibiotics in their management (1,2).

Among recent antibiotics, the one most active against the staphylococcus has been erythromycin, but this organism has been shown to acquire resistance quite rapidly as a result of exposure to erythromycin, both *in vitro* and during the therapy of patients(1,3, 4). Carbomycin, another antibiotic subsequently made available, was also shown to develop almost complete cross-resistance to erythromycin *in vitro*(5-7). Additional antibiotics with considerable *in vitro* activity against *M. aureus* have been developed more recently, and some of them have already received some clinical trials. The present paper presents results of studies on development of resistance and cross-resistance of *M. aureus in vitro* to 3 of these new agents, namely spiramycin(8-14), oleandomycin (PA-105)(15, 16) and streptogramin ("Antibiotic 900") (17), and these, in turn, were tested for cross-resistance to erythromycin and carbomycin.‡ The effect of exposures to these 5 antibiotics on susceptibility of the strains to almost all commonly used antibiotics and to some additional new ones was also tested.

Materials and methods. Four strains of *M. aureus* were used, 2 of them (strains A and B) were highly sensitive to penicillin and the tetracyclines, whereas the other 2 (strains C and D) were highly resistant to them. All 4 were originally inhibited by moderately low concentrations of each of the 5 antibiotics under study. The strains were grown on the surface of a series of plates containing heart infusion agar (Difco, pH 7.2 ±) in which graded concentrations of the antibiotics were incorporated. The original inoculum was derived from a freshly grown overnight culture of the strain in broth and this was streaked on to the agar with a 2 mm loop. The confluent growth from the surface of the agar containing the greatest concentration of antibiotic on which growth was similar to that occurring without antibiotic was used for the next passage to a similar series of plates containing the same antibiotic; this growth was scraped off with a larger loop and emulsified in 0.5 ml of broth and the resulting suspension used for the transfer. The process was repeated until full growth occurred on plates containing 400 μg/ml, the largest concentration used in this study, and was then usually transferred a number of additional times on this concentration of the agent. After each 3 subcultures on one of the antibiotics, the inoculum for the next subculture was also used for tests on similar series of plates containing the other 4 antibiotics in order to determine the extent of the cross-resistance that had developed. At the completion of the study, all of the parent cultures, which had been subcultured in parallel on antibiotic-free agar, and those that had been serially exposed to each of the 5 antibiotics were tested, in addition, for sensitivity to 10 antibiotics, including 6 that are in com-

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* Aided by grant from National Institutes of Health.

† With technical assistance of Clare Wilcox and Ann Najarian.

‡ Spiramycin and streptogramin were furnished by Sharp & Dohme Division, Merck and Co., the Oleandomycin was supplied by Parke-Davis Co. and also by Chas. Pfizer and Co., the latter also furnished the carbomycin (Magnamycin), and the erythromycin (Ilotycin) was obtained from Eli Lilly and Co.

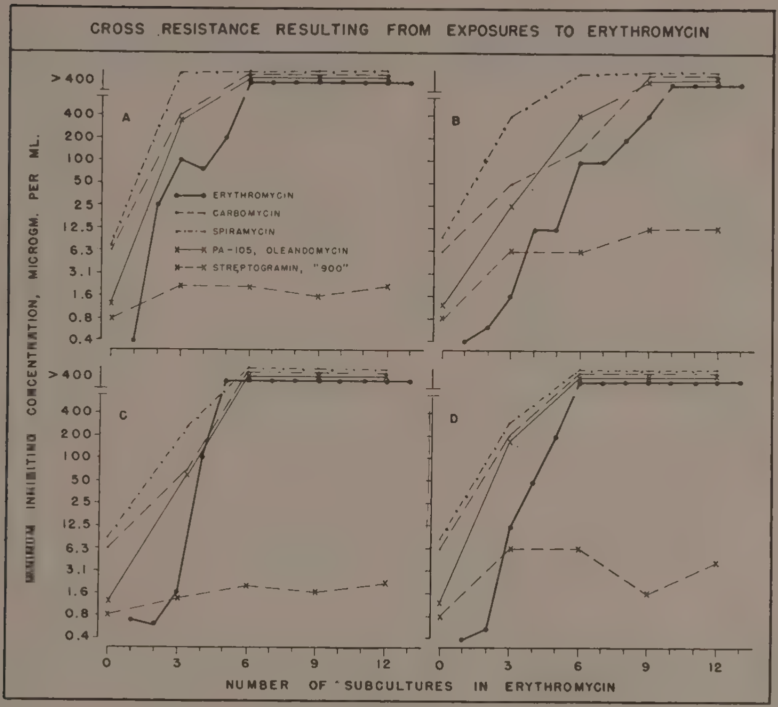

FIG. 1.

mon use and 4 that are currently under investigation.

Results. The minimum inhibiting concentration (M.I.C.) of erythromycin for each of the 4 strains[§] after each successive transfer on that antibiotic, and the susceptibility to the other 4 antibiotics of these strains after each third subculture is shown in Fig. 1. All 4 strains were originally inhibited by 0.4 μg/ml of erythromycin. Each of them developed resistance to the erythromycin very rapidly in the presence of that antibiotic. Strain C grew uninhibited on agar containing 400 μg/ml of that antibiotic after 5 subcultures, Strains A and D after 6 and Strain B after 10 such exposures. Cross-resistance to 3 of the other antibiotics developed at about the same rate, as evidenced by the similar slope of the curves in the figures. The M.I.C. of each of the parent strains for these 3 antibiotics (spiramycin, carbomycin and oleandomycin) was 2 to 8 times that for erythromycin, and the number of transfers required to render the strains resistant to 400 μg/ml of these agents was somewhat greater. Streptogramin was the exception; only a slight decrease in susceptibility of the strains to this antibiotic occurred after the first few exposures to erythromycin but there was no further increase in cross-resistance after further transfers on erythromycin.

Resistance of the same strains to carbomycin resulting from the repeated exposures to that antibiotic developed at a variable rate in the different strains, most rapidly in Strain C (Fig. 2). Cross-resistance to the other antibiotics resulting from these exposures to carbomycin, varied in a similar manner, and developed either at the same rate or at somewhat slower rate. Cross-resistance to spiramycin occurred with particular rapidity, at a rate equal to or even exceeding that against the homologous antibiotic; however, the par-

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[§] The strains are designated A,B,C, and D corresponding to the letters shown in the panels of the text figures.

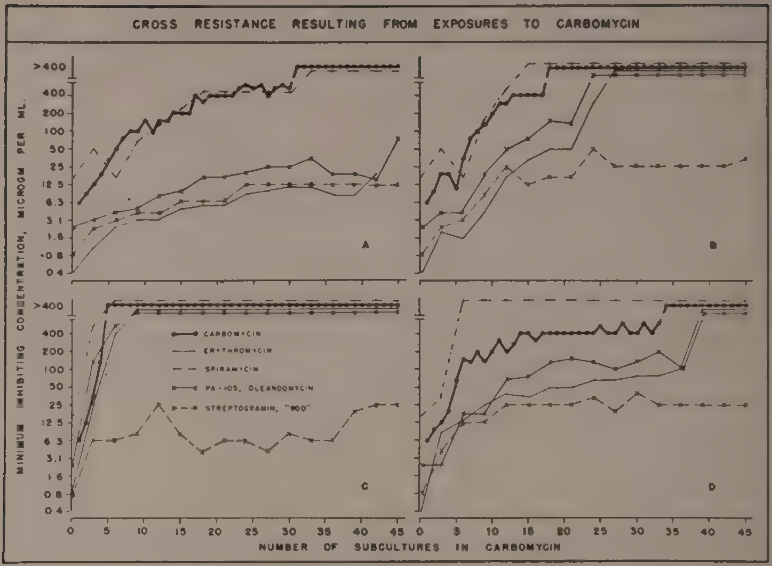

FIG. 2.

ent strains were also somewhat less susceptible to spiramycin than to carbomycin. The reverse was true with respect to the other 3 antibiotics; the parent strains were somewhat more susceptible and they developed cross-resistance at the same or somewhat slower rate, and more subcultures were required before the strains acquired cross-resistance to 400 μg/ml. For Strain A this degree of resistance was not reached after a total of 45 subcultures, or 15 transfers after this strain had acquired that degree of resistance to both carbomycin and spiramycin. Cross-resistance against streptogramin occurred to a somewhat greater extent than after exposure to erythromycin and this tended to level off after the first few exposures to carbomycin just as it did after the exposures to erythromycin.

Exposures to spiramycin (Fig. 3) resulted in the development of resistance to the homologous antibiotic and cross-resistance to the others at about the same rate as was observed with erythromycin. However, only Strain A showed any significant cross-resistance to streptogramin and this appeared to be transient.

Oleandomycin also induced resistance to the homologous agent and to all of the others, except streptogramin, quite rapidly and uniformly. There was no change whatever in the M.I.C. of the 4 strains to streptogramin during the 12 subcultures on oleandomycin (Fig. 4).

Exposures of the strains to streptogramin (Fig. 5), resulted in the development of resistance to both the homologous antibiotic and to the other 4 at a slower rate than was shown in the previous figures. From 32 to 43 subcultures on streptogramin were required to increase the resistance of the strains so that they grew in the presence of 400 μg/ml of that agent. Cross-resistance to the other agents developed at about the same rate and somewhat more slowly to erythromycin.

After these observations were completed, all of the parent strains which had been transferred in parallel on the same kind of agar without antibiotics, were again tested for their sensitivity to the 5 antibiotics used in the study and these, together with the strains that had been exposed and became resistant to the 5 antibiotics were tested for sensitivity to the following additional antibiotics: penicillin, streptomycin, neomycin, tetracycline, chloramphenicol and bacitracin, and also to 4 new antibiotics: bryamycin, cycloserine, albamy-

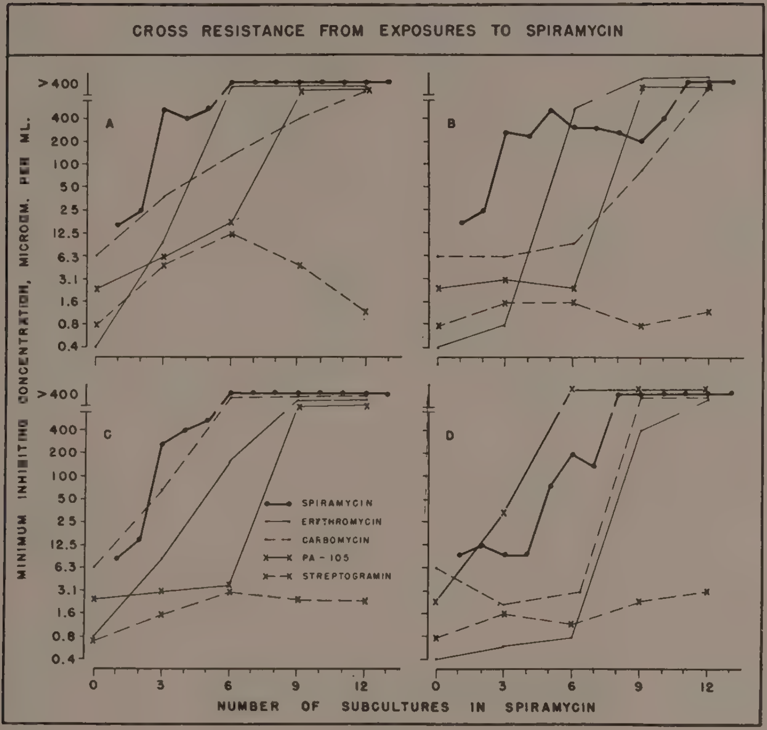

FIG. 3.

cin and vancomycin. The strains had retained their original degree of susceptibility to the 5 antibiotics during their repeated subculture on the agar without the antibiotics, and both these strains and the 5 series of resistant strains derived from them, exhibited the same susceptibility, within the limits of the method, to each of the other antibiotics. In other unpublished studies from this laboratory, strains made resistant by repeated subcultures in cycloserine, bryamin, albamycin and vancomycin showed no cross-resistance to each other, nor to any of the 5 antibiotics that were the subject of the present study, nor to the other antibiotics that are in common use. Resistance to vancomycin developed at a rate even slower than was shown here for streptogramin.

Discussion. The need for effective agents to combat the increasing number and severity of staphylococcal infections still offers a great challenge even now, with such active antibiotics as penicillin, the tetracyclines and erythromycin. These agents are still highly effective so long as the staphylococci in the lesions remain susceptible, but the nature of the staphylococcal lesion, like that in tuberculosis, is particularly favorable for the development and persistence of resistant variants. The organisms persist, often intracellularly, in necrotic and well walled-off lesions, rendering them relatively inaccessible to the full impact of systemically administered antibiotics; as a result, the staphylococci may increase in resistance and become even more difficult to eliminate. Moreover, staphylococci are readily available from the environment to contaminate superficial wounds and to spread from carriers who harbor them as saprophytes but in whom resistant variants may be selected out and then continue to be carried during antibiotic treatment or prophylaxis against other infections. This situation emphasizes the need for a highly effective

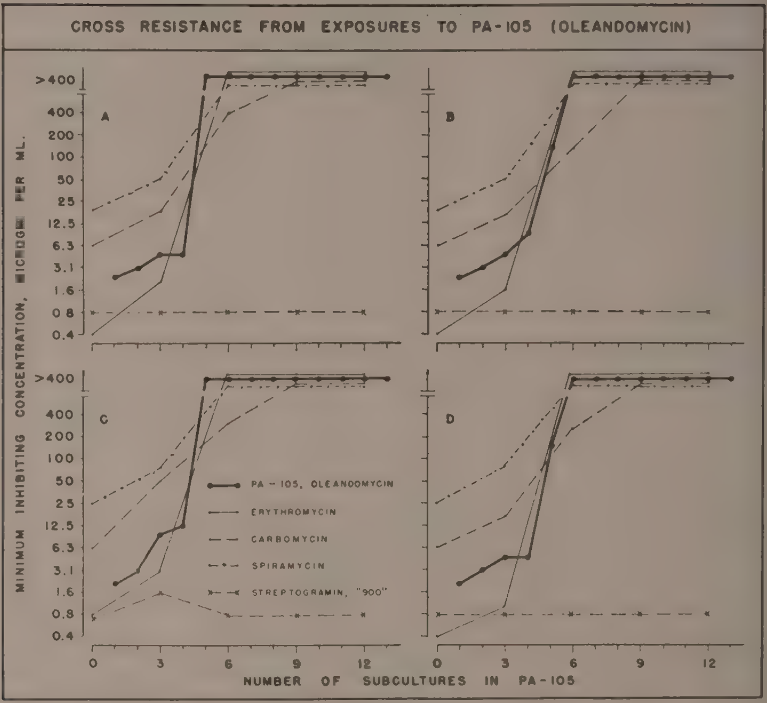
CROSS RESISTANCE FROM EXPOSURES TO PA-105 (OLEANDOMYCIN)
MINIMUM INHIBITING CONCENTRATION, MICROGM PER ML.
>400
400
200
100
50
25
12.5
6.3
3.1
1.6
0.8
0.4
A
B
C
D
PA-105, OLEANDOMYCIN
ERYTHROMYCIN
CARBOMYCIN
SPIRAMYCIN
STREPTOGRAMIN, "900"
0
3
6
9
12
NUMBER OF SUBCULTURES IN PA-105

FIG. 4.

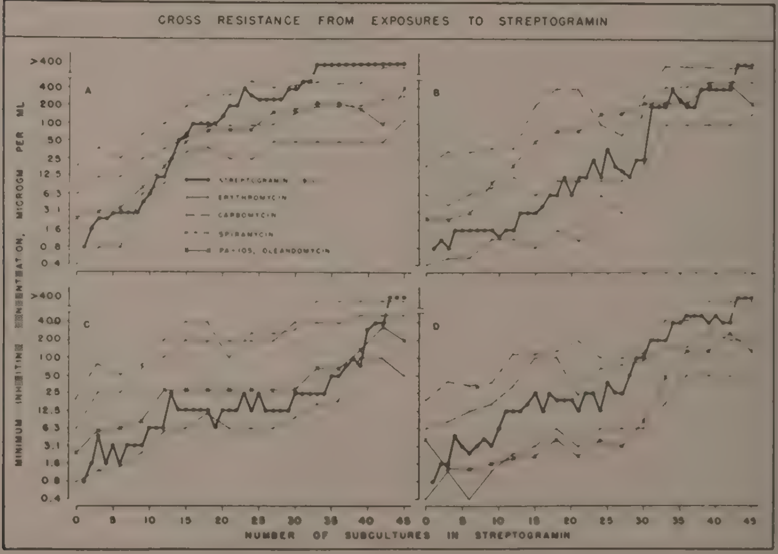
CROSS RESISTANCE FROM EXPOSURES TO STREPTOGRAMIN
MINIMUM INHIBITING CONCENTRATION, MICROGM PER ML
>400
400
200
100
50
25
12.5
6.3
3.1
1.6
0.8
0.4
A
B
C
D
STREPTOGRAMIN
ERYTHROMYCIN
CARBOMYCIN
SPIRAMYCIN
PA-105, OLEANDOMYCIN
0
5
10
15
20
25
30
35
40
45
NUMBER OF SUBCULTURES IN STREPTOGRAMIN

FIG. 5.

bactericidal agent, with good penetrating power, which can be used for long periods systemically, and which lacks the property of inducing resistance in staphylococci.

The present study demonstrated that at least 4 of the 5 agents that were investigated readily induced the development of resistant variants in the strains of staphylococci. There were some differences in the rate at which such resistance developed, depending on the antibiotic, and to a less extent on the strain. Moreover, cross-resistance developed at a rate comparable to that of the homologous resistance except against streptogramin; this antibiotic induced resistance more slowly than did the other 4 agents and, in turn, showed little tendency to lose its activity against the strains that had acquired resistance to those other agents. The possibility that vancomycin may be even more favorable in this respect is suggested by our own unpublished observations which corroborate those of others(18).

The data presented here were obtained *in vitro* and are valid for the conditions of the study but may not necessarily hold for all conditions of exposure of the bacteria to the antibiotics *in vitro*, and certainly may not be true *in vivo*. Ross, for example found 22 strains from patients that were resistant to erythromycin but were either sensitive, or at least moderately so to oleandomycin(19). Chabbert(10), however, found cross-resistance to erythromycin, carbomycin and spiramycin in every strain isolated from patients who had been treated with erythromycin. Observations made in this laboratory on erythromycin-resistant strains isolated from patients,‖ indicated variable degrees of cross-resistance to carbomycin and many strains of staphylococci showed none, although such cross-resistance has been observed regularly after exposures to these agents *in vitro*(5-7). However, nearly 70% of the erythromycin-resistant strains were also resistant to oleandomycin and more than one-fourth of them were resistant to spiramycin.

Summary and conclusions. Four strains of *M. aureus* were rendered resistant to erythromycin, carbomycin, oleandomycin, spiramycin and streptogramin by repeated subcultures in the presence of increasing concentrations of these antibiotics. Cross-resistance to about the same degree developed to all of these agents except to streptogramin; the latter produced homologous resistance at a slower rate than the others, but this was associated with a corresponding increase in cross-resistance to the other 4 antibiotics.

‖ Obtained from Dr. Mark H. Lepper.

1. Finland, M., *New England J. Med.*, 1955, v253, 903, 969, 1019.

2. Wise, R. I., Cranny, C., and Spink, W. W., *Am. J. Med.*, 1956, v20, 176.

3. Haight, T. H., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 183.

4. Finland, M., and Haight, T. H., *Arch. Int. Med.*; 1953, v91, 143.

5. Finland, M., Wilcox, C., Wright, S. S., and Purcell, E. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 725.

6. Fusillo, M. H., Noyes, H. E., Pulaski, E. J., and Tim, J. Y. S., *Antibiotics and Chemother.*, 1953, v3, 581.

7. Holson, D., *Brit. M. J.*, 1954, v1, 236.

8. Pinnert-Sindico, S., Ninet, L., Peu d'Homme, J., and César, C., *Antibiotics Annual*, 1954-1955, p724.

9. Pellerat, J., and Murat, M., *Presse Med.*, 1955, v63, 9 and 166.

10. Chabbert, Y., *Ann. Inst. Pasteur*, 1955, v89, 434.

11. *Idem., ibid.*, 1956, v90, 787.

12. Lepper, M. H., *et al., Antibiotics Annual*, 1955-1956, p658.

13. Ravina, A., *et al., ibid.*, p223.

14. Hudson, D. G., Yoshihara, G. M., and Kirby, W. M. M., *Arch. Int. Med.*, 1956, v97, 57.

15. Sobin, B. A., English, A. R., and Celmar, W. D., *Antibiotics Annual*, 1954-1955, p82.

16. Ross, S., *ibid.*, 1955-1956, p600.

17. Charney, J., *et al., ibid.*, 1953-1954, p171.

18. Ziegler, D. W., Wolfe, R. N., and McGuire, J M., *ibid.*, 1955-1956, p612.

19. Ross, S., *Antibiotics Annual*, 1955-1956, p600.

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In vivo Release of Histamine from Rabbit Blood by Reserpine. (22767)

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Reserpine is a widely employed and useful drug in the treatment of a variety of clinical conditions, particularly hypertension and certain psychiatric disorders. One of the most common and consistent side effects of reserpine therapy in man has been nasal stuffiness. This effect may be relieved in most patients by the use of vasoconstrictive substances and in some by oral medication with antihistaminics(1). In addition, reserpine has been found to cause an increase in the volume and free acidity of gastric secretion following both oral and intravenous administration of this drug to humans(2). These secondary findings suggested the possibility that reserpine could cause the release of histamine from its body sources. Recent studies have shown that reserpine liberates serotonin (5-hydroxytryptamine) from its depots in animals(3), and from human platelets *in vivo*. Both histamine and serotonin are present in rabbit blood platelets in relatively large amounts as compared to other amines. Previous studies had indicated that reserpine incubated *in vitro* with rabbit platelets caused a release of platelet serotonin but no histamine release.

The following study was done to determine whether or not reserpine liberated histamine *in vivo* from rabbit blood.

Methods and materials. Male rabbits (1.5-2.0 kg) were given a single intravenous injection of reserpine (5 mg/kg). A control blood specimen was taken prior to the injection, and experimental samples at various intervals after the injection. All samples were drawn by cardiac puncture into siliconed glassware using heparin as anticoagulant. Both histamine and serotonin were determined chemically on whole blood. For each determination, 1 ml of whole blood was diluted to 8 ml with water, 1 ml of 10% zinc sulfate solution added and, after shaking, 0.5 ml of 1 N sodium hydroxide. Following centrifugation at high speed, a 1 ml aliquot of the supernatant was analyzed directly for serotonin by measuring its specific fluorescence in strong acid(4). Five ml of the remaining supernatant were analyzed for histamine employing modifications of the methods of McIntire *et al.* (5,6) and Lowry *et al.*(7). The details of the methods employed here, whereby both serotonin and histamine can be determined on the same supernatant after protein precipitation will be reported later. Recoveries, standards and blanks were run along with each determination. For both serotonin and histamine, recoveries were over 80%. The rabbit whole blood values obtained in this work compare favorably with those previously reported for histamine(6,8) and serotonin.*

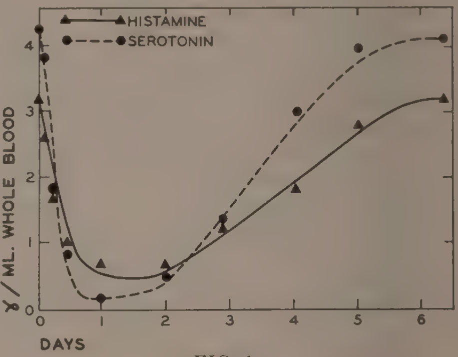

FIG. 1.

Results. Composite data from 5 animals given reserpine are shown in Fig. 1. The reduction in whole blood histamine was similar in rate and degree to the reduction in whole blood serotonin. The return of histamine and serotonin values to control levels was also parallel. The curves for each individual animal were substantially the same, reaching a minimal level at about 20 hours, remaining low until about 48 hours, and returning to normal in 5 to 7 days. The minimum histamine level at 40 hours varied from 7% to 25% of the control value in the animals. All the rabbits showed typical reserpine effects. Previous

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* Unpublished observations.

TABLE I. Effect of Reserpine on Rabbit Blood, and Platelet Serotonin and Histamine.

	Serotonin		Histamine	
	Control	24 hr	Control	24 hr
Whole blood, γ/ml*	4.6	.5	3.0	.6
Platelet, γ/mg protein†	6.8	.7	3.7	.7

* Avg of 5 animals. † Avg of 2 animals.

work has shown that platelet counts are essentially the same in rabbits before and 16 hours after the intravenous injection of 5 mg/kg of reserpine.* Since in the rabbit both histamine and serotonin are found in platelets, the decrease in whole blood histamine and serotonin cannot be accounted for by a reduction in whole blood platelet count. Instead, a release from platelets is postulated with subsequent metabolism of both serotonin and histamine to account for the reduction of these constituents in whole blood.

In support of a release phenomenon to account for the drop in whole blood histamine and serotonin, platelets were isolated according to the method of Dillard *et al.*(9) from rabbit whole blood before and again 24 hours after an intravenous injection of reserpine. Both histamine and serotonin were determined and related to the amount of platelet protein(10) as shown in Table I.

Discussion. It is well substantiated that reserpine liberates serotonin from the body depots in animals, and from platelets in man. In the study presented here, histamine has been shown to be liberated from rabbit blood *in vivo*. Recently Carlsson(11) has shown epinephrin release from rabbit adrenals by reserpine, but further work* has shown that this release does not occur in a cord sectioned animal which indicates an indirect effect. These studies suggest that reserpine is capable of liberating several potent biologically active substances from blood and tissues, although its action may not necessarily be a direct one.

Histamine is known to cause the release of catechol amines(12), particularly in patients with pheochromocytomas, and in turn is released by a variety of primary amines(13,14), including epinephrine(15,16). Feldberg and Smith(17) have shown the release of histamine from tissues by serotonin using bioassay. It is therefore possible that these compounds are mutually able to release each other from blood and tissues although perhaps to a variable degree and extent. Reserpine may be capable of a primary liberation of histamine, serotonin and epinephrine, or by liberating one of these cause a secondary release of the others. That reserpine may liberate other substances also becomes a possibility. Finally, one can speculate that the therapeutic effects as well as the side effects of this drug may be due to the release of these basic compounds, their attendant interaction, and resultant loss. However, preliminary experiments indicate that reserpine does not release histamine from other animal tissues *in vivo*.

Studies on the release of histamine from tissues in animals, and blood in humans are in progress in this laboratory.

Summary. Intravenous reserpine in rabbits has been demonstrated by chemical means to release histamine from whole blood. 1. The minimal histamine levels were reached in one to 2 days with complete recovery to control values in 5 to 7 days. 2. Simultaneous serotonin determinations, revealed that the rate and degree of liberation, and the recovery of both histamine and serotonin were very similar. 3. Platelets, isolated 24 hours after reserpine, showed a marked reduction in both histamine and serotonin compared to control platelets.

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1. Wilkins, R. W., *Essential Hypertension, Disease-A-Month,* Yearbook Publishers, Inc., Feb. 1955.

2. Haverback, B. J., Stevenson, T. D., Sjoerdsma, A., and Terry, L. L., *Am. J. Med. Sc.,* 1955, v230, 601.

3. Pletscher, A., Shore, P. A., and Brodie, B. B., *J Pharm. and Exp. Therap.,* 1956, v116, 84.

4. Udenfriend, S., Bogdanski, D. F., and Weissbach, B., *Science,* 1955, v122, 972.

5. McIntire, F. C., Roth, L. W., and Shaw, J. L., *J. Biol. Chem.,* 1947, v170, 537.

6. McIntire, F. C., White, F. B., and Sproull, M., *Arch. Biochem.,* 1950, v29, 376.

7. Lowry, O. H., Graham, H. T., Harris, F. B, Priebat, M. K., Marks, A. R., and Bregman, R. V., *J. Pharm. and Exp. Therap.,* 1954, v112, 116.

8. Rosenthal, S. M., and Tabor, H., *ibid.,* 1948, v92, 425.

9. Dillard, G. H., Brecher, G., and Cronkite, E. P.,

PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 853.

10. Sutherland, E. W., Cori, C. F., Haynes, R., Olsen, N. S., *J. Biol. Chem.*, 1949, v180, 825.

11. Carlsson, A., *Kungl. Fysiografiska/Söllskoepts I Lund Forbandlingar* BD 26 NR 8, 1956.

12. Roth, G. M., and Kvale, W. F., *Am. J. Med. Sc.*, 1945, v210, 653.

13. MacIntosh, F. C., and Paton, W. D. M., *J. Physiol.*, 1949, v109, 190.

14. Mongar, J. L., *ibid.*, 1952, v119, 48P.

15. Eichler, O., and Barfuss, F., *Arch. Exp. Path. Pharmak.*, 1940, v140, 156.

16. Staub, H., *Helv. Physiol. Pharmacol. Acta*, 1946, v4, 539.

17. Feldberg, W., and Smith, A. N., *Brit. J. Pharm. and Chemother.*, 1953, v8, 406.

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Electrophoretic Behavior of Some Animal Hemoglobins. (22768)

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The authors have been unable to find any report of results of paper electrophoresis of hemoglobins other than human. The present communication presents findings in 6 species of animals.

Methods. Electrophoresis was performed on "sandwich" type plate glass apparatus using Whatman 3MM paper in sheets 36 × 16 cm and 36 × 12 cm. Barbiturate buffer at pH 8.6 and M 0.025 was used in all preliminary experiments. Current was applied to 36 × 16 cm sheets at 300 volts and 5 milliamperes for 6 hours and to 36 × 12 cm sheets for 4 hours, constant temperature of 20°C was maintained. Under these conditions normal human hemoglobin (Hb. A) migrated approximately 5 cm during the run. In addition to electrophoresis alkali denaturation tests(1) and solubility tests(2) were carried out on all specimens (Table I). Rat hemoglobin was obtained from the pooled blood of adult Wistar rats killed at the end of experiments. Dog hemoglobin was from mongrel dogs used in experimental surgery. Rabbit hemoglobin was from albino rabbits used for pregnancy tests. Chicken hemoglobin was from pooled blood obtained from a poultry farm. Turtle hemoglobin was from several specimens of the common land turtle, *Gopherus polyphemus*. Alligator hemoglobin was from a single small specimen of *Alligator missippiensis*. Hemoglobin solutions were prepared in the usual manner and adjusted to approximately 5%, 3 lambda of the solution were applied to the paper.

TABLE I. Resistance to Alkali Denaturation and Solubility as COHb of Animal Hemoglobins.

Species	% alkali resistant	% soluble as carbonmonoxy-hemoglobin
Dog	0	100
Rabbit	36	"
Rat	100	"
Chicken	35	"
Alligator	0	"
Turtle	0	"

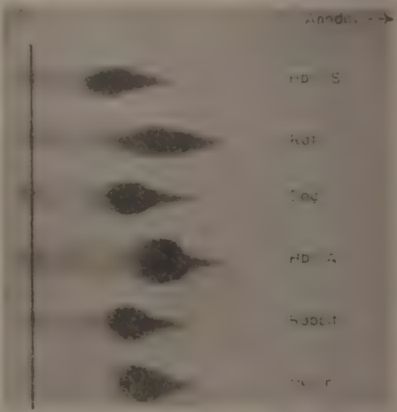

FIG. 1. Patterns of rat, dog and rabbit hemoglobins with Hb. S., Hb. A. and Hb. F. for comparison.

Results. As shown in Fig. 1, dog and rabbit hemoglobins move as homogenous compounds at a rate slightly slower than fetal hemoglobin but faster than Hb. S while rat hemoglobin produces a trailing spot suggesting a mixture of hemoglobins; this we have been unable to resolve by varying the buffer or any other factors.

In Fig. 2, turtle and chicken hemoglobin behave as mixtures, these were fairly well separated by electrophoresis in phosphate buffer at pH 7.4 (Fig. 4). Alligator hemoglobin (Fig. 3) moves at a slightly more rapid rate than Hb. A and presents a trailing spot which does not resolve into 2 components at pH 7.4.

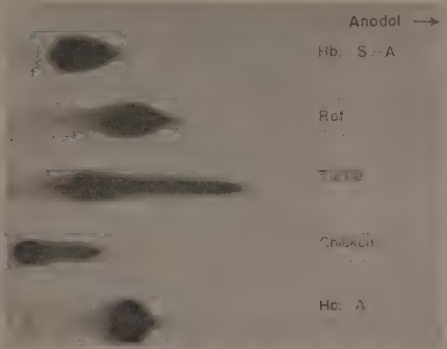

FIG. 2. Patterns of turtle and chicken hemoglobins with rat, Hb. S-A and Hb. A for comparison.

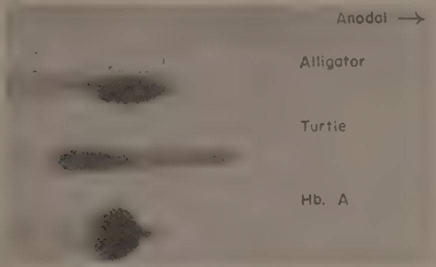

FIG. 3. Patterns of alligator and turtle hemoglobins with Hb. A for comparison.

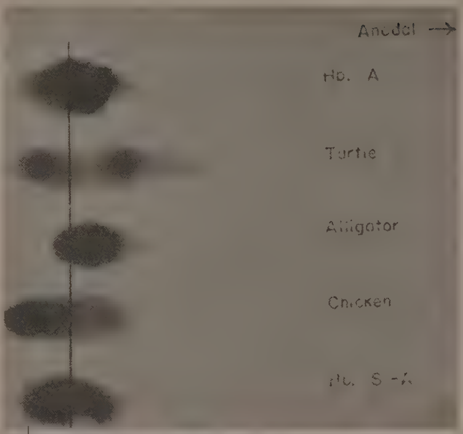

FIG. 4. Patterns of turtle, alligator and chicken hemoglobins run in phosphate buffer at pH 7.4, 150 volts, 5 milliamperes for 6 hr. Hb. A and Hb. S-A for comparison.

These observations are preliminary and incomplete in that many methods of examination of hemoglobins have not yet been employed. They are presented in the hope that they may be of interest to zoologists and perhaps phylogenists.

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1. Singer, K., Chernoff, A. I., and Singer, L., *Blood*, 1951, v6, 47.

2. Itano, H. A., *Arch. Biochem.*, 1953, v47, 148.

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Production of Fatty Livers by 4-Aminopyrazolo-(3,4-d)-Pyrimidine. Toxicological and Pathological Studies.* (22769)

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Recently interest has been aroused in the possible use of the pyrazolopyrimidines as potential chemotherapeutic agents for the treatment of human neoplasms. Robins(1) synthesized a series of such compounds with the hope that the isomers of the naturally-occurring purines might be active. Subsequently the adenine isomer, 4-aminopyrazolo-(3,4-d)-pyrimidine (4-APP), was shown by Skipper (2) to be the most active of the series in inhibiting the mouse mammary adenocarcinoma 755 and in increasing the life span of mice with L1210 or L5178 leukemia. The results indicated that 4-APP might be worthy of clinical trial and accordingly pharmacological studies were undertaken in laboratory animals.

Procedure. Mice, rats, cats, and dogs were employed. The mice in all cases were male Swiss albino (Millerton Research Farms). Male CFW (Carworth Farms Wistar) rats were used for the toxicological studies while the pathological studies were carried out with male CFN rats (Carworth Farms Nelson) (3,4). The latter are Wistar rats which have been bred free of chronic murine pneumonia. Most of the toxicological, hematological, biochemical, and pathological methods employed herein have been described(5,6). As the 4-APP was found to be hepatotoxic, alkaline phosphatase in plasma was added to other previously described tests of liver function in dogs. The procedure of Bodansky with the modification of Gomori(7,8) was employed for this purpose. For administration of animals the agent was solubilized in H_2O by the addition of molar equivalent amounts of HCl.

TABLE I. Toxicity of 4-APP.

Species	Route of admin.	No. of successive daily doses	LD_{50}	19/20 confidence limits*	S*
			←mg/kg/day→		
Mice	Intraper.	1	290	260–320	1.11
	”	5	25	18– 34	1.8
	Oral	1	180	116–279	2.4
	”	5	12.5	11– 15	1.20
Rats	Intraper.	1	228	127–410	1.54
	”	5	17.5	10– 32	2.21
	Oral	1	141	108–185	1.42
	”	5	18	15– 20	1.10
Cats	Intrav.	1	50–100	—	—
Dogs	Intrav.	10†	1.25–2.5	—	—
	Oral	10†	1.25–2.5	—	—

* Calculated according to Litchfield and Wilcoxon(9). Animals were observed for at least 2 wk after end of treatment.

† Maximum No. of doses given daily excepting weekends.

Toxicity in rodents. In Table I are presented LD_{50} data in mice and rats. 4-APP was shown to be of the same order of toxicity in both species and to be as toxic, or possibly more toxic, when given by the oral route than when injected intraperitoneally. The most prominent finding in the rodents given high single doses either by mouth or intraperitoneally was the appearance within two hours of blepharostenosis, blepharospasm and hypersensitivity. Of those given 400 mg/kg intraperitoneally all of the rats and most of the mice died unobserved overnight; the remaining mice lost weight progressively and died 12 to 13 days later. Single lethal doses given orally to mice (equal to or less than 400 mg/kg) resulted in delayed deaths between 3 and 14 days after intubation. In rats oral doses of similar magnitude caused death between 1 and 4 days. Weight loss was the only consistent finding associated with the delayed

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The 4-aminopyrazolo-(3,4-d)-pyrimidine was kindly supplied by Dr. R. K. Robins, New Mexico Highlands University.

fatalities; an occasional animal appeared unkempt or showed diarrhea. Rats and mice which had received 4-APP on each of 5 successive days by either route of administration showed progressive weight loss beginning 24 hours after the first dose. Such animals became unkempt and occasionally the rats had "bloody" exudates about the nares. Most of the deaths occurred between the fifth and seventh days after initiation of treatment.

Pathological changes in rats. The lesions associated with intoxication in rats were studied in the CFN strain. For this purpose an initial group of 8 animals was injected intraperitoneally with 4 successive daily doses of 25 mg/kg/day. At 96 hours after initiation of this treatment the animals showed only a moderate weight loss and appeared less intoxicated than desired for the pathological studies. Accordingly they were not sacrificed and all eventually regained their lost weight and survived. (It is interesting to note that in the CFW strain this dose had proved to be greater than the LD_{50}. The lesser susceptibility of the CFN strain in this instance is in keeping with our recent experience comparing the toxicity of various agents in both kinds of Wistar rat.) A second group of CFN rats was then injected with 50 mg/kg/day for 4 days. Six of these were sacrificed at 96 hours at which time all showed severe weight loss and an untidy, unthrifty appearance. The remaining 2 rats were dead by the seventh day. The *gross findings* in all the 6 sacrificed animals were: an average weight loss of 20%, pale livers, small spleens and pallor of the renal cortex. Two showed dark purple adrenals. On microscopic examination a fat nephrosis was found in the kidneys as evidenced by slight vacuolization in the epithelium of proximal convoluted tubules. The vacuoles were primarily subnuclear and were shown to contain fine drops of fat in frozen sections stained with Sudan IV. A fine, diffuse cytoplasmic vacuolization was also seen in hepatic cells and shown to be fat by the Sudan IV stain. Except for the fatty change the livers appeared normal; there was no evidence of necrosis, inflammation or bile duct involvement. The sternal bone marrow showed an 85-90% loss of nucleated elements. In 2 animals recent cortical hemorrhages were present in the adrenals and in one of this pair the medulla was similarly involved. The adrenals of a third animal showed only medullary hemorrhages. In all 6 animals the red pulp of the spleen was reduced in amount and, though the thymus was involuted, the other lymphoid tissues showed no significant microscopic changes. No alterations were observed in the intestines or in the other viscera studied.

Toxicity in cats. Three respective pairs were given single intravenous doses of 100, 50, and 25 mg/kg. The highest dose caused immediate urination, salivation, hyperpnea and panting, a sluggish pupillary reflex, and relaxation of the nictitating membrane. At 5 minutes one of the pair given this dose had slight convulsive movements and was unable to stand. Both cats vomited several hours after injection and at 6 hours, though hyperpnea and salivation had ceased, the animals appeared moderately depressed. Thereafter depression and ataxia were evident until the animals died, one during the second day and the other during the third day. Except for retching and vomiting at 3 to 4 hours after 50 mg/kg the two lower doses produced no significant signs of intoxication and all 4 recipients survived the 3-week period of observation.

Toxicity in dogs. Each of the following lethal doses were tested in a pair of animals: 10, 5, and 2.5 mg/kg/day intravenously and 5 and 2.5 mg/kg/day by mouth. The 2 given 10 mg/kg/day died at 5 and 6 days after initiation of treatment; 7 of the remaining 8 died between 7 and 16 days; the 1 survivor had received 5 mg/kg/day by mouth. During the course of intoxication weight loss, anorexia, and vomiting appeared in all animals between the second and fifth days after initiation of treatment. Small ulcerations of the buccal mucosa were noted in 2 of the 10 dogs while 2 others showed patches of slight congestion in this area. Increases in fluid intake and in urine output with a concomitant fall in the specific gravity of urine (to 1.010 or less) were noted in 8. Nine developed loose stools by the third day and 4 of these showed tarry stools with evidence of blood by the end of the first week. Four others had tarry stools on the day of death. Jaundice was present at 7 to 10 days (as evidenced by a yellow cast in the sclera and buccal mucosa) in 2 of the animals given 2.5 or 5 mg/kg/day intravenously. There was no jaundice seen in any of the dogs treated orally with 4-APP. Body temperatures remained normal throughout the course of fatal intoxication except for hypothermia 24 hours before death in the 2 given 10 mg/kg/day intravenously. Pairs of animals were also given doses of 1.25 mg/kg/day and 0.63 mg/kg/day, either orally or intravenously; all of these dogs survived and showed no untoward signs.

Pathological changes in dogs. Table II presents hematological and biochemical findings in a group of 4 dogs treated with intravenous doses of 4-APP. Dogs 1 and 2 received 5 mg/kg/day for 4 consecutive days while dogs 3 and 4 were given 9 successive daily injections of 2.5 mg/kg/day excepting weekends. All 4 animals were sacrificed 24 hours after the last injection. The course of intoxication in each was like that described above.

All developed reticulocytopenia and a depression of the nucleated cell count in bone marrow as seen in samples aspirated from the iliac crest. In 3 of the animals hemoconcentration was present at the time of sacrifice; in dog 4 an increased hematocrit was seen at 7 days. Significant lymphocytopenia was noted in all dogs; conversely 3 showed a neutrophilic granulocytosis. Dogs 3 and 4, which had been subjected to the more prolonged course of intoxication, had decreased numbers of circulating platelets by the eleventh day. Both dogs receiving the higher dose (5 mg/kg/day) had an obvious defect in blood coagulation as evidenced by the marked prolongations of clotting times (Table II). This disturbance was found to be associated with deficiencies of 2 of the components of the blood clotting mechanism. In Table II it will be seen that prothrombin times became greatly elevated and simultaneously the fibrinogen contents of plasma decreased significantly. Dogs 3 and 4 which had received 2.5 mg/kg/day shown no significant changes in clotting time but did develop increased pro-

TABLE II. Hematological and Biochemical Findings in Dogs Given 4-APP.

Dog	Day	Wt (kg)	Hcrit. (ml/100 ml)	Retic. (%)	PMN-N	Lcyte	Pltlt.	BMNC	Clot time (min.)	Fbgen	Blrbn	BSP	Prot. time (sec.)	Alk. ptase (mg %)
					(10³ cells/mm³)					(mg %)				
1	0	12.3	57	.1	7.8	1.4	134	170	5.8	216	.3	.2	13.6	.3
	4†	10.2	77	0	22.3	.2	192	24	>22.0	68	1.2	2.8	52.1	4.7
2	0	10.7	47	.1	5.2	1.1	190	126	3.8	305	.2	.3	12.4	2.5
	4	9.2	56	0	21.6	0	164	16	22.0	*	3.9	—	>120	7.8
3	0	9.8	52	.2	14.6	1.1	432	211	4.5	—	(1)	.1	15.4	(2)
	7	7.9	61	0	10.5	.2	262	24	6.8	—	—	—	16.3	—
	11†	7.1	59	0	13.6	2.1	106	—	8.5	—	.5	1.3	35.2	2.1
4	0	15.9	47	.8	8.8	2.3	206	224	5.0		(1)	.2	12.6	(2)
	7	13.7	53	0	9.6	3.8	218	61	5.3			.3	12.1	—
	11	13.5	47	0	16.1	.7	34	41	6.8		.4	.8	18.1	3.6

Abbreviations: Alk. ptase, plasma alkaline phosphatase; Blrbn, total plasma bilirubin; BMNC, bone marrow nucleated cells; BSP, bromosulfalein retention in blood; Clot., clotting; Fbgen, plasma fibrinogen; Hcrit, hematocrit; Lcyte, lymphocytes; Pltlt, platelets; PMN-N, polymorphonuclear neutrophils; Prot., one-stage prothrombin; Retic., reticulocytes.

* The oxalated plasma failed to clot upon addition of $CaCl_2$ and thrombin. † Dogs 1 and 3 were moribund at sacrifice.

(1) Avg control value for 49 dogs = .2 ± .1; (2) avg control value for 30 dogs = 1.3 ± .9.

thrombin times. No plasma fibrinogen determinations were done in these dogs.

Dogs 1 and 2 had significant increases in plasma bilirubin. In dogs 1, 3 and 4 in which measurements were made on the day of sacrifice bromosulfalein (BSP) retention was found to be elevated. The extent of this elevation in dogs 1 and 3 may have been exaggerated by the concomitant hemoconcentration and moribund state of these animals. Changes in plasma alkaline phosphatase were not impressive.

In additional biochemical analyses, which are not recorded in Table II, plasma protein and blood glucose remained relatively constant. Non-protein nitrogen in plasma rose from initial values of 15-25 mg % to 32-59 on the day of sacrifice. Dogs 2 and 3 which had developed hemoconcentration also showed significant decreases in plasma chloride (from 116 and 113 to 100 and 105 mEq/l respectively; unfortunately this analysis was not carried out in plasma from dog 1.

On *microscopic examination* the most consistent findings were in the livers of all 4 dogs. In dogs 1 and 2 which had been given the higher daily doses of 4-APP a fine diffuse cytoplasmic vacuolization was present in the hepatic cells; in frozen sections Sudan IV stain revealed this to be fat. In addition both livers showed plugging of the bile canaliculi; one had a slight cholangiolitic proliferation. In neither animal was there evidence of any necrotic or inflammatory changes in the liver. Other significant findings in dogs 1 and 2 were recent hemorrhages in the adrenal cortices of both and in the medulla of one. One of this pair also had hemorrhages in the myocardium, lungs, and small and large intestines. The bone marrows of both were congested but the nucleated elements appeared normal. Dogs 3 and 4 which had received the lower daily doses also had fatty livers but to a lesser extent than did 1 and 2. Again neither necrotic nor inflammatory cells were present; in this pair abnormalities were not noted in the bile ducts and canaliculi. As in dogs 1 and 2 sections of vertebral and sternal bone marrow revealed marked congestion but again the number of nucleated elements was within normal limits. No pertinent changes were seen in the kidney, intestine, spleen, or any of the other viscera studied.

Discussion. Of the purine and purine analogs which have been studied toxicologically (10) 4-APP, an isomer of adenine, is the only one known to cause fatty livers in rats and dogs although 2,6-diaminopurine has been found to induce fatty metamorphosis in pigs (11). The present study has shown that in dogs the fatty change may be accompanied by stasis of bile in the canaliculi and by evidence for hepatic insufficiency, that is, elevations in the one-stage prothrombin time and decreases in the fibrinogen content of plasma (12,13). Such changes appear in conjunction with and probably account for the blood coagulation defect. Jaundice and increased plasma bilirubin may also be seen; the hyperbilirubinemia may be related to the intrahepatic biliary stasis.

The hepatic changes produced by 4-APP can be distinguished from those caused by a close analog, 6-mercaptopurine(5,10). The latter induces hepatic necrosis in mice and rats without fatty alteration. In dogs the same agent produces stasis of bile in canaliculi similar to, but more extensive than, that caused by 4-APP; there is no evidence for fatty metamorphosis. Interestingly, the changes in tests of hepatic function in dogs intoxicated with 6-mercaptopurine are those expected to result from biliary obstruction: hyperbilirubinemia, increased retention of BSP, and marked rise in plasma alkaline phosphatase. Prothrombin time, however, remains unaltered(10).† In contrast the prominent sign of hepatic insufficiency resulting from 4-APP is the disturbance of the prothrombin complex.

Microscopic study revealed no significant change in the kidneys of dogs though fat nephrosis was observed in lethally intoxicated rats. These observations are in contrast to those previously reported with adenine, 2-chloroadenine and purine(10). Intoxication of rats with the last-mentioned purines is characterized by the deposition of occlusive crystals in the renal tubules.

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† Unpublished.

Rats given 4-APP also showed a significant decrease in the content of nucleated cells in the bone marrow. In intoxicated dogs, though bone marrow aspirated from the iliac crest contained decreased numbers of nucleated elements, microscopic study of decalcified sections revealed marked congestion in the marrow capillaries but neither decreases nor abnormalities in hematopoietic elements. It is possible that the aspirated marrow appeared depleted due to dilution with blood from the congested capillaries.

Adrenal cortical hemorrhages were found in several of the rats and dogs which had been sacrificed for pathological study. The significance of these lesions is obscure; but their presence is interesting in light of the finding that 4-APP also induces hypochloremia and polyuria in dogs.

Summary and conclusions. The toxic effects of 4-aminopyrazolo-(3,4-d)-pyrimidine (4-APP) have been studied in mice, rats, cats, and dogs. The agent was found to produce a fatty change in liver which was associated neither with necrosis nor with inflammation. Hepatic insufficiency was evidenced by hyperbilirubinemia, increases in one-stage prothrombin time and decreases in plasma fibrinogen. In rats fat nephrosis and depletion of hematopoietic elements in bone marrow were also seen. Hepatic insufficiency should be anticipated as a possible hazard in clinical trials with 4-APP.

1. Robins, R. K., *J. Am. Chem. Soc.*, 1956, v78, 789.

2. Skipper, H. E., Robins, R. K., and Thomson, J. R., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 954.

3. Cummings, C. N. W., *Carworth Farms Quarterly Letter*, 1956, no. 40, New City, Rockland Co., N. Y.

4. Nelson, J. B., *J. Exp. Med.*, 1951, v94, 377.

5. Clarke, D. A., Philips, F. S., Sternberg, S. S., Stock, C. C., Elion, G. B., and Hitchings, G. H., *Cancer Research*, 1953, v13, 593.

6. Philips, F. S., Sternberg, S. S., Hamilton, L. D., and Clarke, D. A., *Cancer*, 1956, v9, No. 6.

7. Bodansky, A., *J. Biol. Chem.*, 1933, v101, 93.

8. Gomori, G., *J. Lab. Clin. Med.*, 1941-42, v27, 955.

9. Litchfield, J. T., Jr., and Wilcoxon, F., *J. Pharmacol. Exp. Therap.*, 1949, v95, 99.

10. Philips, F. S., Sternberg, S. S., Hamilton, L., and Clarke, D. A., *Ann. New York Acad. Sc.*, 1954, v60, 283.

11. Cartwright, G. E., Palmer, J. G., Hitchings, G. B., Elion, G. B., Gunn, F. D., and Wintrobe, M. M., *J. Lab. Clin. Med.*, 1950, v35, 518.

12. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, v66, 801.

13. Sykes, E. M., Jr., Seegers, W. H., and Ware, A. G., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 506.

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Demonstration of a Marked Bence-Jones Proteinemia.* (22770)

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Previous reports of Bence-Jones proteinemia have been founded upon a variety of observations: precipitate formation in multiple myeloma sera when heated to 55-60°C(1-3); aberrant serum protein solubility characteristics on salt fractionation(4); undemonstrated but postulated identity of a crystalline substance from a myeloma serum with crystals in the corresponding Bence-Jones protein containing urine(5); appearance of a new or elevated serum peak in electrophoretic pattern which closely corresponds in mobility to that of urinary Bence-Jones protein(4); immunological cross reaction with and blocking of antibody to Bence-Jones protein by the myeloma serum(6); and demonstration of slow sedimenting serum components which are electrophoretically in the γ- and β-globulin areas(6).

Most of these criteria can not be considered

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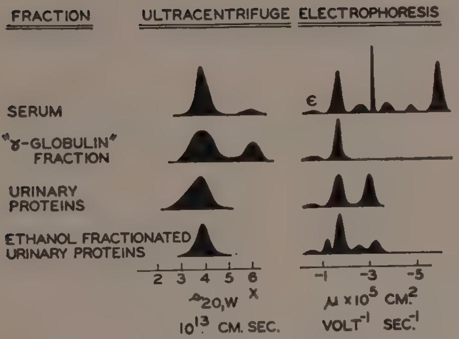
FRACTION
ULTRACENTRIFUGE
ELECTROPHORESIS
SERUM
"γ-GLOBULIN" FRACTION
URINARY PROTEINS
ETHANOL FRACTIONATED URINARY PROTEINS
ε
2 3 4 5 6
$s_{20,w}$ x 10^{13} CM. SEC.
−1 −3 −5
μ x 10^5 CM^2 VOLT^{-1} SEC^{-1}

FIG. 1. Ultracentrifuge and electrophoresis diagrams of the serum and urinary proteins and fractions thereof from a case of multiple myeloma with Bence-Jones proteinemia.

in themselves adequate evidence of serum Bence-Jones protein, although the above experiments strongly suggest its presence. In this study, analysis of a multiple myeloma serum and fractions thereof by a combination of electrophoretic and ultracentrifugal technics has clearly demonstrated a considerable amount of Bence-Jones protein. This serum entity has properties in common with one of the major protein components of the urine.

Experimental and results. A patient with clinically diagnosed multiple myeloma had a total serum protein of 8.9% and an A/G ratio of 0.94 as determined by Howe fractionation. Electrophoretic analysis of the serum in pH 8.6, 0.1 ionic strength diethylbarbiturate buffer showed an increased γ-globulin area comprising 26% of total protein. The electrophoretic and ultracentrifuge diagrams of this serum and other protein systems studied, are presented in Fig. 1. The ultracentrifuge analysis of the serum carried out in the Spinco apparatus at 59,780 r.p.m. in pH 7.4, 0.2 ionic strength potassium phosphate buffer evidenced three components. These consisted of the usual small amount of an 18 S component and 2 other slower sedimenting boundaries. Only the latter 2 are seen in Fig. 1. The minor of these had an $s_{20,w}$ near 6 S and comprised 16% of the total. The slower peak had an average sedimentation constant of 3.87 S. The small amount of the 6 S component of this serum was surprising in view of the enhanced γ-globulin level (26% as compared to normal serum level of near 12%). This result suggested that the elevated γ-globulin area might also contain considerable amounts of a low molecular weight protein.

The serum was fractionated by cold ethanol technics(7) to remove the γ-globulin fraction. Electrophoretic examination of the "purified" γ-globulin fraction (see Fig. 1) revealed a single component with a mobility of −1.8 x 10^{-5} cm^2 volts^{-1} sec.$^{-1}$ at pH 8.6. It is represented by a single symmetrical electrophoretic peak at pH values from 4.6 to 8.6. The isoelectric point was 5.9.

Ultracentrifugal analysis of this material, however, showed that 65% of it consisted of protein sedimenting with the velocity of a Bence-Jones protein (8,10). Its $s_{20,w}$ was 3.87 S and showed no concentration dependency from 0.47 to 3.0%. The faster component sedimented with a velocity of 6.02 S and thus appears to be a γ-globulin. The yield data indicated that the slower sedimenting material was present in the serum at a level near 1.4% and thus comprised near 16% of the total serum proteins.

The patient evidenced an "albuminuria" of about 10 g/day. Electrophoretic examination of the dialyzed urine and of the urinary proteins insoluble in 50% saturated ammonium sulfate revealed 2 main components. One of these components had electrophoretic mobility of a β-globulin while the other was near that of serum γ-globulin peak. Data for electrophoretic mobility of the latter urinary component and serum γ-globulin fraction as a function of pH is plotted to give Fig. 2. It is apparent that the charge relationships of the

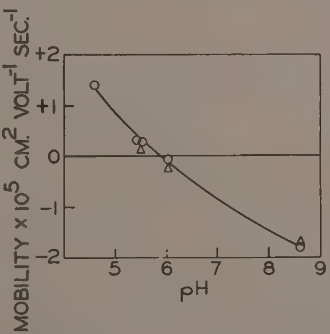

FIG. 2. Electrophoretic mobility of the serum "γ-globulin" fraction –○–○– and the major urinary component –△–△– as a function of pH.

protein from blood and urine are quite similar.

Ultracentrifuge analysis of the urinary proteins revealed a broad peak, with about 35% of the material sedimenting as a slow shoulder of the main component. The latter sedimented near 3.8 S.

The urinary proteins insoluble in 50% saturated ammonium sulfate were precipitated twice with ethanol (pH 7.0, 20% ethanol, 0.01 ionic strength, –6°C) in an attempt to purify the component with electrophoretic mobility of a γ-globulin. The resultant material when examined in the ultracentrifuge showed none of the slow shoulder characteristic of the crude urinary protein (Fig. 1). The sedimentation constant was 3.87 S, which is the same as that obtained for the low molecular weight portion of the serum γ-globulin fraction. The electrophoretic pattern (Fig. 1) shows that the minor urinary protein components had been concentrated.

Neither urinary nor serum proteins nor fractions thereof evidenced the typical Bence-Jones protein heat precipitation reaction. They coagulated on heating to near 70°C and failed to redissolve at 100°C. Filtrates of the boiled material did not give any precipitate on cooling. Nevertheless, their size and charge properties, their association with clinically diagnosed myeloma and their presence in the urine indicate that these are Bence-Jones proteins. Analogous heat reactions of such proteins have been encountered previously(9,10).

Discussion. The above results offer conclusive evidence that a protein corresponding to urinary Bence-Jones proteins may be present in certain myeloma sera. Attention should be called to the fact that a combination of fractionation, electrophoretic, and ultracentrifuge experiments on both serum and urine are required to clearly show this. Previous work using various single methods of study have not been entirely convincing. Shortcomings are present in conclusions based on electrophoretic similarity of serum components to Bence-Jones proteins without concurrent sedimentation studies being carried out. Immunological cross-reaction and blocking between the lower molecular weight Bence-Jones protein and the serum globulin components may be so strong that distinction by this means is not possible(11).

The serum protein studied here showed no concentration dependency of its sedimentation constant and would thus appear to be a relatively symmetrical molecule possessing a molecular weight of 35,000 to 40,000(9,10). The ability of this small molecule to accumulate in the serum is surprising. Previous studies in the dog have indicated that molecules of this size are cleared through the kidney quite rapidly(12). Furthermore, most myeloma patients with Bence-Jones proteinuria do not appear to show a significant Bence-Jones proteinemia(3,6).

It might be pointed out that the relatively high incidence of "albuminuria" reported in the condition of multiple myeloma is likely due to Bence-Jones proteins of the heat coagulable type described in the present and in other reports(9,10).

Summary. Simultaneous electrophoretic and ultracentrifugal studies of the serum proteins of a multiple myeloma patient have conclusively demonstrated a considerable Bence-Jones proteinemia. The properties of the serum component correspond to those of one of the major urinary components.

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1. Perlsweig, W. A., Delrue G., and Geschickter, C., *J.A.M.A.*, 1928, v90, 755.

2. Cantarow, A., *Am. J. M. Sc.*, 1935, v189, 425.

3. Collier, F. C., Reich, A., and King, J. W., *New Eng. J. Med.*, 1951, v245, 969.

4. Gutman, A., Moore, D., Gutman, E., McClellan, V., and Kabat, E., *J. Clin. Inv.*, 1941, v20, 765.

5. Packalen, T., *Acta med. Scandinav.*, 1939, v100, 1.

6. Moore, D. H., Kabat, E. A., and Gutman, A. B., *J. Clin. Inv.*, 1943, v22, 67.

7. Nichol, J. C., and Deutsch, H. F., *J. Am. Chem. Soc.*, 1948, v70, 80.

8. Svedberg, T., and Pedersen, K., *The Ultracentrifuge*, Oxford University Press, 1940.

9. Deutsch, H. F., *J. Biol. Chem.*, 1955, v216, 97.

10. Putnam, F. W., and Stelos, P., *J. Biol. Chem.*, 1953, v203, 347.

11. Deutsch, H. F., Kratochvil, C. H., and Reif, A. E., *ibid.*, 1955, v216, 103.

12. Marshall, M. E., and Deutsch, H. F., *Am. J. Physiol.*, 1950, v163, 461.

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Distribution and Partial Purification of Pituitary Gonadotropins of Human Plasma.* (22771)

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The presence of a substance or substances possessing gonadotropic activity, presumably of pituitary origin, in the serum of ovariectomized women was first demonstrated by Fluhmann(1). Subsequently, Cohn and his collaborators(2) reported the detection of two gonadotropic hormones in fractions prepared from certain pools of human plasma. Since these findings could not be fully confirmed in later studies,[†] we have reexamined the distribution of pituitary gonadotropic activity among various plasma fractions. The bulk of the gonadotropic activity has been located in Fraction II + III, and by subfractionation has been partially purified. Preliminary reports of these studies have appeared elsewhere (3,4).

Methods and materials. Postmenopausal plasma was employed exclusively in this study, preliminary bioassays having shown that gonadotropic activity is readily demonstrable in the whole plasma of postmenopausal women, but not in that of men or of adult women of menstrual age. The donors were healthy women 65 years of age or less who were not undergoing treatment with estrogenic preparations of any kind.[‡] The blood was collected either with citrate or through cationic exchange resins in the ADL-Cohn blood fractionating machine. The data herein reported were obtained by fractionating a 10-liter plasma pool which comprised the donations of 40 women, and are identical with the results previously obtained from the fractionation of 20 individual or pooled plasma samples from 3-12 donors. The cold ethanol method VI described by Cohn *et al.* (6) was employed for the initial plasma fractionation. The following fractions were prepared: Fraction I containing the fibrinogen; II + III consisting of β-lipoproteins and gamma globulin; IV-1 containing the α-globulins and cholesterol; IV-4 consisting of α- and β-globulins and the metal-combining globulin; V, which contains the bulk of the albumin; and VI, the supernatant remaining after fractionation. Partial purification of the gonadotropically-active material in Fraction II + III was accomplished by precipitating the bulk of the biologically-inactive proteins with zinc. Fraction II + III was brought to the original plasma volume with 0.15 M sodium chloride and zinc glycinate added until a 5 mM zinc concentration was obtained at a pH of 6.8 and a temperature of 0°C. The supernatant was freed of zinc by dialysis against ethylene diamine tetra-acetic acid (EDTA) and sodium citrate. The EDTA and citrate were subsequently removed by dialysis against 0.15 M sodium chloride. The addition of 20% ethanol to the purified supernatant at a pH of 6.5 and a temperature of —5°C resulted in the precipitation of additional material which was biologically inactive, leaving the gonadotropins in a supernatant containing 2-4% of the proteins originally present in the fraction. In preparation for bioassay the fractions were redissolved in small volumes of 0.15 M NaCl and dialyzed for 24 hours against 0.15 M NaCl in order to remove residual alcohol and excess salt. All samples were stored at 2°C and their pH adjusted to 7.0 ± 0.4 immedi-

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* This work was supported by grants from Barbara C. Willcox Fund of Vincent Memorial Hospital, Amer. Cancer Soc. (Mass. Division), and by research grant RG-4444 from National I.H., P.H.S.

[†] Unpublished data.

[‡] Previous experience has shown that unwitting ingestion of estrogen occasionally results in significant contamination of plasma pools. Therefore, the whole plasma and those fractions found to produce an increase in uterine weight of intact rats were assayed for estrogen in spayed rats by modification of method of Bulbring and Burn(5). No indication of estrogenic activity was found.

TABLE I. Relative Gonadotropic Activity of Whole Postmenopausal Plasma and of Its Individual Fractions.

Preparation	% plasma proteins	Plasma equivalence (ml)	No. of animals	Mean wt, mg* Uterus	Mean wt, mg* Ovary
Whole plasma	100.0	15	3	24.6	17.2
		20	3	*91.5*	14.0
		40	6	*110.1*	*24.6*
I	4.1	400	3	15.2	11.3
II + III	25.0	15	2	23.3	14.8
		20	3	*39.7*	16.7
		40	3	*97.3*	16.1
		80	3	*131.4*	*21.8*
IV-1	7.5	400	3	23.9	15.9
		500	3	*58.6*	12.1
		666	3	*146.5*	15.7
IV-4	11.6	550	3	26.3	14.7
		700	3	*125.5*	18.8
V	50.0	600	3	24.9	18.9
		750	2	*73.0*	18.5
VI	1.0	700	3	33.2	16.9

* Mean uterine weights in excess of 38.3 and mean ovarian weights in excess of 21.1 mg are regarded as differing significantly from control weights ($p < 0.01$) and are italicized.

ately prior to assay. "Gonadotropic activity," which presumably comprises the action of two substances, pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH), was assayed biologically. Immature female rats of the Sprague-Dawley strain, weighing 45-55 g, were injected subcutaneously with the material to be tested twice daily for 4 days and were killed on the fifth day. The wet weights of the ovaries and the fluid-free uterus were employed as indices of gonadotropic activity, the minimum quantity of whole plasma and of the individual fractions required to induce statistically significant increases in the weights of these organs being ascertained. In 23 control animals, the mean ovarian weight was 15.1 ± 3.7 and the mean uterine weight 28.2 ± 6.2 mg. Therefore, that volume of plasma which just sufficed to increase the mean ovarian weight of the three test animals above 21.1 or the mean uterine weight above 38.3 mg was regarded as the minimal effective dose ($p<0.01$).

Results. The relative gonadotropic activity of whole plasma and of its various fractions is shown in Table I. The gonadotropic potency of whole plasma ($\text{M.E.D.}_{\text{ut. wt.}}$, 20 ml; $\text{M.E.D.}_{\text{ov. wt.}}$, 40 ml) accords well with Fluhmann's observations(1). Employing as his test animal the immature female mouse, which is approximately 6 times as sensitive to human menopausal gonadotropin as the rat (7), Fluhmann demonstrated gonadotropic activity in 3-5 ml of the serum of ovariectomized women. The finding that appreciably greater gonadotropic stimulation is required for ovarian growth than for the induction of ovarian secretory activity is in agreement with many previous observations.

The bulk of the gonadotropic activity is seen to be concentrated in Fraction II + III, although traces of activity are demonstrable in Fractions IV-1, IV-4 and V. The potency of Fraction II + III would appear to be somewhat more than half that of whole

TABLE II. Relative Gonadotropic Activity of Fraction II + III, and of Its Subfractions.

Preparation	% proteins in Fraction II + III	Plasma equivalence (ml)	No. of animals	Mean uterine wt (mg)*	Mean ovarian wt (mg)*
II + III	100	20	3	*39.7*	16.7
		40	3	*97.3*	16.1
		80	3	*131.4*	*21.8*
5 mM Zn^{++} precipitate	94	300	3	35.7	14.1
" supernatant	6	20	3	26.3	18.2
		40	3	*126.1*	18.1
		80	3	*141.9*	*35.3*
20% ethanol precipitate from 5 mM Zn^{++} supernatant	3	100	3	25.8	14.7
20% ethanol supernatant from 5 mM Zn^{++} supernatant	3	40	2	*98.8*	15.4
		80	3	*115.7*	*34.9*

* Mean uterine weights in excess of 38.3 and mean ovarian weights in excess of 21.1 mg are regarded as differing significantly from control weights ($p < 0.01$) and are italicized.

plasma, whether gauged by the $M.E.D._{ut.\ wt.}$ or the $M.E.D._{ov.wt.}$ Fractions I and VI appear to be devoid of gonadotropic activity.

The relative gonadotropic potency of Fraction II + III and of its subfractions is shown in Table II. It will be noted that zinc precipitation effectively frees the gonadotropically-active material from the majority of the contaminating proteins and that re-precipitation with 20% ethanol removes additional contaminants.

Summary. The location and approximate concentration of gonadotropic activity in various fractions of human postmenopausal plasma has been determined. The plasma was fractionated by means of the cold ethanol method VI of Cohn and was assayed in immature female rats by estimating ovarian growth and secretory activity. Although traces of activity were found in Fractions IV-1, IV-4 and V, the bulk of the gonadotropically-active material was located in Fraction II + III. Subfractionation of Fraction II + III by successive precipitations with zinc and 20% ethanol freed the gonadotropins from the majority of the contaminating proteins.

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1. Fluhmann, C. F., *J.A.M.A.*, 1929, v93, 672.
2. Cohn, E. J., Oncley, J. L., Strong, L. E., Hughes, W. L., Jr., and Armstrong, H. S., Jr., *J. Clin. Invest.*, 1944, v23, 417.
3. Pennell, R. B., 9th Conf. on plasma proteins and cellular elements of the blood. Protein Foundation, 1954, Nov. 15, pp45-47.
4. Antoniades, H. N., Ingersoll, F. M., and McArthur, J. W., 10th Conf. on plasma proteins and cellular elements of the blood. Protein Foundation, 1956, Jan. 5-6.
5. Bulbring, E., and Burn, J. H., *J. Physiol.*, 1935, v85, 320.
6. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J.A.C.S.*, 1946, v68, 459.
7. Evans, J. S., Hines, L. R., Varney, R., and Koch, F. C., *Endocrinology*, 1940, v26, 1005.

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SECTION MEETINGS

CLEVELAND, O. University Hospital	October 15, 1956
DISTRICT OF COLUMBIA George Washington University	October 4, 1956
ILLINOIS Michael Reese Hospital	October 9, 1956
OHIO VALLEY University of Cincinnati	October 6, 1956

Ultra-Violet Flying Spot Television Microscopy. Study of Living HeLa Cells.[*] (22772)

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In another communication the authors have described a technic for obtaining relatively monochromatic U-V television images by means of the flying spot microscope(1). This system is in operation, and the purpose of this paper will be to report results of preliminary experiments designed to establish the period of time over which unchanged images can be obtained with the flying spot system.

Methods. The light source is a U-V emitting cathode ray scanner tube on the face of which is formed a 240 line raster. This raster is traced by a spot deflected horizontally at 60 cycles/second and vertically at one cycle every 4 seconds. These sweep rates were arbitrarily chosen to investigate potentialities of the system. A minified image of this raster is formed on the specimen by a Bausch and Lomb .75 NA Grey reflecting objective. Transmitted radiant energy is collected by a reflecting condenser optically matched to the objective and is focused on the entrance slit of a Bausch and Lomb grating monochromator. An ultra-violet-sensitive photomultiplier tube is located at the exit slit of the monochromator. The monochromator is adjusted to 2680 Angstroms with a 100 Angstrom band width. The electrical output of the multiplier tube is amplified and modulates a monitor display tube. The time bases of this monitor tube are locked in synchronism with those of the scanner tube. The screen has a very long persistency, of the order commonly employed for radar tubes. This long persistency screen is necessary because of the long vertical sweep cycle. Thus, the entire system becomes an

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[†] Dept. of Pathology, Southwestern Medical School.

[‡] Visiting scientist Southwestern Medical Foundation.

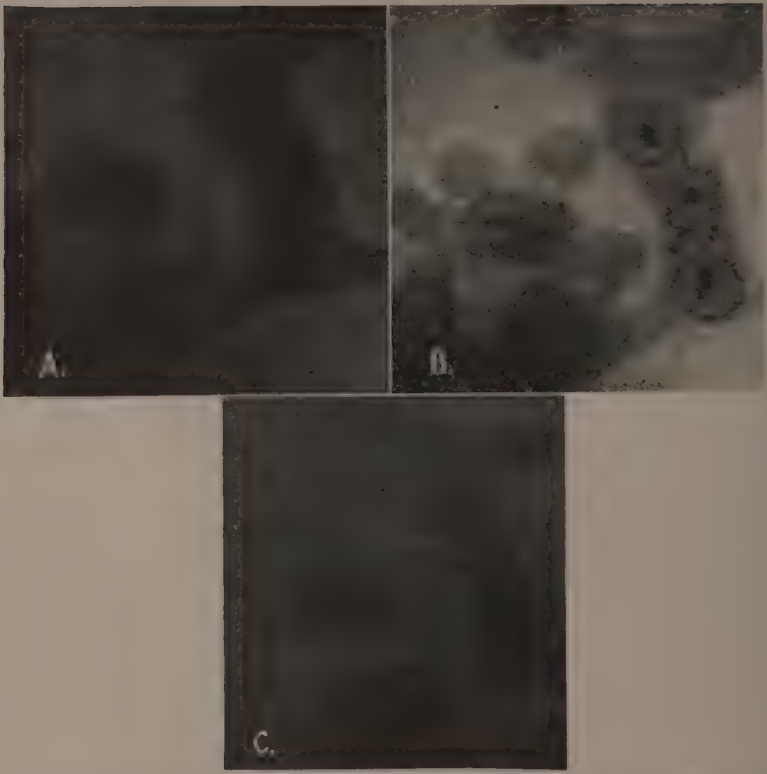

FIG. 1. Living HeLa cells at 2680 Å (667×). A. Original appearance. B. presence of cytoplasmic appearance at the end of 6 hr of continuous scanning. C. Return to normal after 12 hr interruption in scanning.

image converter and a specimen scanned in ultra-violet is displayed in visible light. The image on the monitor screen is photographed by a Polaroid Land camera. The signal to noise ratio of the display at these low radiation levels requires that the images of several consecutive frames be stored in the film emulsion. The signal to noise ratio then becomes a function of the square root of the number of frames stored. During the entire course of these experiments the microscopic field was continually irradiated by the scanner tube and photographs were obtained at periodic intervals from 15 minutes to 1 hour. In the experiments described, living HeLa strain cells were prepared on Vycor cover slips and sealed to Vycor slides by means of a wax vaseline ring. The cells were suspended in their own media composed of chick embryo extract, human ascitic fluid and Hank's balanced salt solution or in ascitic fluid alone, or in balanced salt alone. We were unable to detect any significant differences in the images obtained attributable to the nature of the media employed. Once the preparation was mounted on the microscope it was allowed to remain at room temperature (72°F) for the duration of the experiment.

Results. Fig. 1A shows a typical absorption photograph of living HeLa cells at 2680 Å as obtained with the flying spot technic. This field was then continuously irradiated and intermittent photographs of the monitor tube were obtained. The photographic images remained the same for a period of approximately 6 hours. At this time cytoplasmic bubbles began to form about the periphery of some of the cells in the field. These bubbles

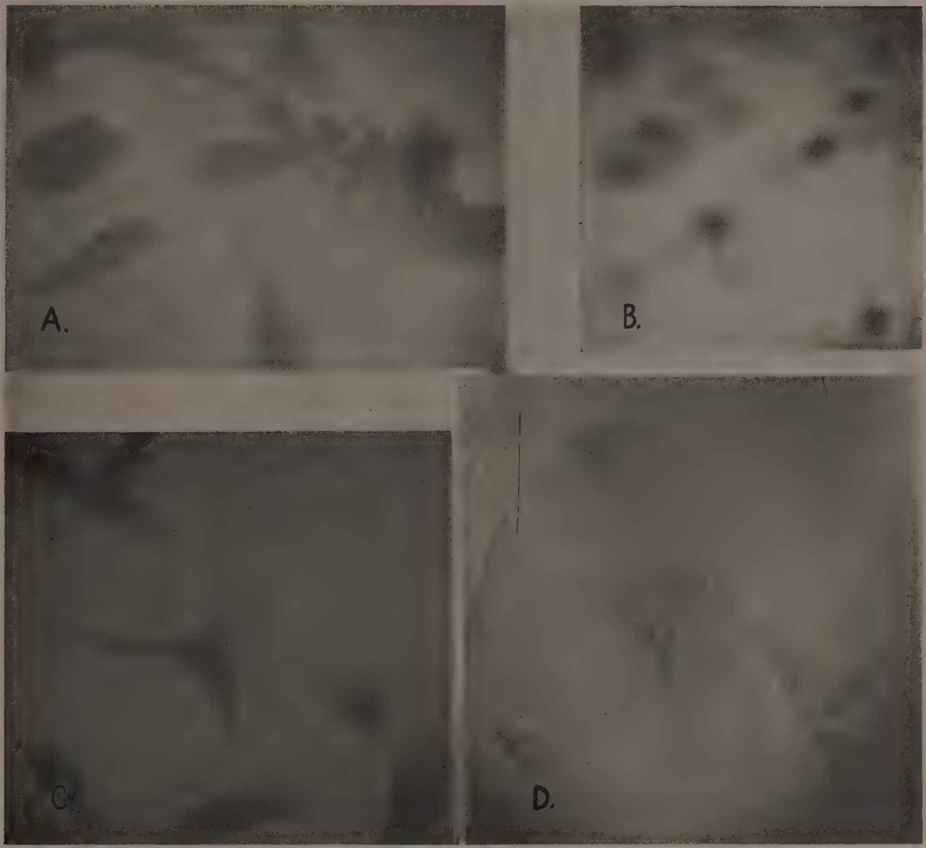

FIG. 2. Living HeLa cells at 2680 Å (533×). A. Original appearance. B. Following 6 hr of continuous scanning showing marked nuclear opacity and cytoplasmic transmission. C. Living HeLa cells at 2680 Å (533×). D. Living HeLa cells at zero orders showing lack of typical U-V absorption.

showed absorption at 2680 Å as may be seen in Fig. 1B but they were clear when viewed with visible light. In this experiment the phenomenon was reversible following a 12 hour respite from the continual irradiation, and the bubbles did not reappear upon subsequent irradiation of 3 hours duration as illustrated by Fig. 1C. One notes that the cytoplasmic absorption, nuclear lack of absorption, nuclear membrane and nucleolus all remain constant in their absorption appearance during this prolonged exposure to U-V irradiation. These 3 photographs do not show identical background densities of the photographic plates in each case and the density of the absorption is thus not quantitatively comparable. The present instrumentation does not permit a constant monitor screen brightness over 64 frames, thus variations in the amount of light reaching the photographic plate are inevitable. In a longer experiment consisting of 7 hours of continuous irradiation marked changes in the absorption pattern of the cells occurred at the end of the 7 hour period. These changes consisted of a complete opacity of the nucleus with lack of definition of the nucleolus, and a relative lack of the normal cytoplasmic absorption. The contrast between the appearance of these cells at the end of 2 hours and at the end of 7 hours is illustrated by Fig. 2A and 2B. In this experiment the cytoplasmic bubbles described in the previous experiment appeared at the end of 5 hours and disappeared just before the appearance of the nuclear opacity.

In a third experiment continuous irradiation was applied to the cells for 6 hours. During this time the photographic images of the monitor screen remained the same. In this experiment 2 photographs were obtained at each time interval. Fig. 2C is a photograph obtained at the end of 2 hours. In this photograph the monochromator is set at 2680 Å. Fig. 2D is a photograph obtained immediately after Fig. 2C, the monochromator in this case is adjusted to the zero orders. Fig. 2C is a typical absorption photograph while Fig. 2D illustrates an image formed principally by light scattering. The latter image shows the characteristic clarity of the living nucleus when viewed with visible light.

Discussion. The principle of the flying spot microscope as described by Roberts and Young(2) has been adapted for use in the deep ultra-violet. The purpose of this adaptation was to find if possible a method which would permit the continuous or nearly continuous recording of ultra-violet absorption images of living cells without producing severe irradiation damage in the process. It would appear from these preliminary studies that this technic will yield just such results.

The failure of each group of cells to respond in an identical fashion to roughly equivalent time intervals of continuous scanning is not explained. It may represent differences in culture technic as the cells were transferred from stock cultures at irregular intervals, or it may represent variations in amount of irradiation. Complete regularity of U-V emission from the scanner tube is not possible in the present state of tube and phosphor development. Furthermore, day to day variations in the room temperature and instruments settings could not be rigidly controlled and it is conceivable that minor uncontrolled variations contributed to the experimental variations observed.

The data presented here are in agreement with those presented by Brumberg and Larionow(3). These authors describe the undamaged nucleus to show little or no absorption at 2680 Å. This is in marked contrast to Caspersson's observations(4), and may perhaps be accounted for by the lack of irradiation damage in this and Brumberg's work. Thus, lack of nuclear absorption by visible or ultra-violet light in normal living cells is one criterion of the absence of significant irradiation damage. As irradiation damage occurs the nuclear absorption increases until the nucleus becomes homogeneously opaque. The apparent increasing density of the nucleus in contrast to its normal transmissiveness may represent an alteration in the physical state of the DNA of the chromosomes. Such alterations have been produced by Ris and Mirsky (5) by physiological saline or fixation procedures. They consider the appearance of structure in the nucleus in visible light to be good evidence for cellular damage.

It appears that the flying spot scanning system of U-V microscopy is capable of producing continuous absorption images of living cells without producing significant irradiation damage. The system may be further improved by several technics. the most important of which will be to make the spot monochromatic before it reaches the specimen.

Summary. 1. Preliminary experiments with the continuous production of monochromatic ultra-violet absorption images of living cells indicate that this may be accomplished by the flying spot television microscope technic.

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1. Montgomery, P. O'B, Roberts, F. F., and Bonner, W. A., *Nature*, 1956, v177, 1172.

2. Roberts, F., and Young, J. Z., *J. Inst. Elec. Eng.*, England, 1952.

3. Brumberg, E. M., and Larionow, L. T., *Nature*, 1946, v158, 663.

4. Caspersson, T., *Skand. Arch. Physiol.*, 1936, Suppl. No. 8.

5. Ris, H., and Mirsky, A. E., *J. Gen. Physiol.*, 1948, v32, 489.

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Inhibition of DOPA Decarboxylase by Aromatic Acids Associated with Phenylpyruvic Oligophrenia. (22773)

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It has been demonstrated(1) that the biochemical lesion associated with mental deficiency in phenylpyruvic oligophrenia is a failure of an enzyme system in the liver of these patients to hydroxylate phenylalanine to tyrosine. This condition results in the appearance of abnormal oxidation products of aromatic amino acids in the blood and urine(2). The light hair and skin color associated with this disease has suggested an impairment of tyrosine metabolism. Experimental support for this suggestion has recently been brought forth in the report, which shows that phenylalanine and the related aberrant aromatic acids associated with this disease, competitively inhibit plant as well as mammalian tyrosinase*(3). Further the repigmentation which has been observed when these patients are placed on a low phenylalanine diet(4) is consistent with this notion.

The recent finding that patients suffering with this affliction have low epinephrine plasma values(5) suggests a further interference with enzyme systems as a consequence of the appearance of abnormal amounts of phenylalanine and its related metabolites. These low plasma levels of epinephrine led us to examine the effects of these aromatic acids on the beef adrenal medulla DOPA decarboxylase, an enzyme which functions in the metabolic pathway for epinephrine synthesis.

Methods. DOPA decarboxylase was obtained from beef adrenal medulla as described by Langemann(6). The tissue was ground with sand in a mortar, suspended in an equal amount of 0.1 M phosphate buffer, pH 6.8 and centrifuged. The rate of decarboxylation was determined with a Warburg respirometer at 37°C. The gas phase was nitrogen. The main compartment contained 0.8 ml enzyme, 40 γ pyridoxal-5-phosphate and inhibitor. One side arm contained 20 µmoles D-L DOPA while the other contained 3.6 N H_2SO_4. After the removal of the air with nitrogen the vessels were closed and equilibrated for 10 minutes. The substrate was tipped into the main compartment and readings were made at 5 and 10 minute intervals. After 10 minutes the acid was tipped in, and a final reading was taken. Sodium phenylpyruvate, phenyllactic acid, and L-phenylalanine were obtained from Nutritional Biochem. Inc., phenylacetic acid was obtained from Matheson, Coleman and Bell Inc.; pyridoxal-5-phosphate was obtained from Mann Biochem. Inc., and D-L dihydroxyphenylalanine was obtained from Eastman Kodak Co.

TABLE I. Inhibition of Adrenal Medulla DOPA Decarboxylase by Phenylketonuria Metabolites.

Additions	Conc., µmoles/3 ml	µl CO_2/hr	% inhibition
Control	—	160	—
Phenylpyruvic acid	100	0	100
	50	0	100
	10	36	77
Phenyl-lactic acid	100	0	100
	50	36	77
	10	82	50
Phenylacetic acid	100	80	50
	50	163	0
L-phenylalanine	100	164	0

Each flask contained 800 mg adrenal medulla, 40 γ pyridoxal phosphate. Phosphate buffer pH 6.8. The gas phase was nitrogen.

Results. The results of these experiments on the inhibition of adrenal DOPA decarboxylase are given in the Table. Phenylpyruvate and phenyllactate were the most effective inhibitors of the series, while phenylacetic acid was poor. L-phenylalanine showed no inhibitory effect. It is interesting to compare these results with those obtained by Hartman *et al.*(7) in their study using DOPA decarboxylase from hog kidney cortex. They obtained comparable inhibitions of their enzyme with phenylpyruvate, and phenylacetic acid, but obtained no inhibitions with phenyllactate.

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* To be published.

These data suggest an explanation for the low plasma levels of epinephrine in patients suffering from phenylpyruvic oligophrenia(5) in that these aromatic acids which accumulate as a result of biochemical lesion may inhibit the synthesis of epinephrine in the adrenal gland.

The importance of epinephrine in normal nerve function is yet to be elaborated; however, its presence in brain tissue is well established(8). Weil-Malherbe has shown that epinephrine plasma levels were significantly lower in a group of mental defectives than in a group of mixed patients(5). As yet, information is not available which would allow us to speculate on the influence of a deficiency of epinephrine on the etiology of mental deficiency.

Summary. Phenylpyruvic acid, phenyllactic acid and phenylacetic acid inhibit beef adrenal medulla DOPA decarboxylase. The implications of these data in phenylpyruvic oligophrenia are discussed.

1. Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 514.

2. Folling, A., *Nord. med. tidskr.*, 1934, v8, 1054. Woolf, L. I., *Biochem. J.*, 1951, v49, LX.

3. Dancis, J., and Balis, M. E., *Pediatrics*, 1955, v15, 63.

4. Armstrong, M. D., and Tyler, F. H., *J. Clin. Invest.*, 1955, v34, 565.

5. Weil-Malherbe, H., and Bone, A. D., *J. Mental Sci.*, 1955, v101, 733.

6. Langemann, H., *Brit. J. Pharm. and Chem.*, 1951, v6, 318.

7. Hartman, W. J., Akawie, R. I., and Clark, W. G., *J. Biol. Chem.*, 1955, v216, 507.

8. Vogt, M., *J. Physiol. (London)*, 1954, v123, 451.

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Stability of the Activator of Bovine Plasminogen. (22774)

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(With Technical Assistance of Ralph E. Clarke)

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Mullertz and Lassen(1) first reported that a mixture of streptokinase and human plasminogen acquired a characteristic which neither the streptokinase nor the plasminogen exhibited alone, namely, the ability to activate bovine plasminogen. They concluded that bovine blood lacks a factor (proactivator) which is present in human blood and suggested that this proactivator combines with streptokinase to form an activator of bovine plasminogen. The studies of Troll and Sherry(2) and Sherry(3) indicated that the activator of bovine plasminogen was also the activator of human plasminogen. It was further demonstrated(2) that the activator which appears after adding streptokinase to human plasminogen was lost by acidification and regained by the re-addition of streptokinase after neutralization of the fraction, indicating that streptokinase was required for activator activity.

This communication calls attention to the observation that a loss of activator can occur during the incubation of a mixture of streptokinase and human plasminogen.* The rate of disappearance of the activator has been found to be affected by the duration of incubation, the initial streptokinase concentration and the particular plasminogen preparation. These observations have significant bearing on the interpretation of the *in vitro* and *in vivo* activation of plasminogen by streptokinase.

Materials. Streptokinase (SK). Varidase (Lederle) was dialyzed before use. Bovine plasminogen was made according to Milstone (4) and then lyophylized. Lysine ethyl ester (LEE) was synthesized according to Werbin and Palm(5) and stored over phosphorous pentoxide. Several fractions of human plasminogen were prepared. The preparations were as follows: #1) A euglobulin precipitate, made from placental blood according to Milstone(4), #2) A lyophylized pooled sample of human plasma, #3) A pH 2.5 acid extract of placental Fr. III_2, prepared as described by Christensen(6) and further purified by an adsorption-elution technic,[†] #4) A Fr. III, obtained as described by Oncley *et al.*(7). These reagents were kept in a desiccator and weighed out immediately before use. A pH 6.4, 0.1 M phosphate buffer was prepared by mixing 300 ml of 0.1 M Na_2HPO_4 with 700 ml 0.1 M NaH_2PO_4, and a pH 7.15 0.1 M phosphate buffer, by mixing 700 ml 0.1 M Na_2HPO_4 and 300 ml of 0.1 M NaH_2PO_4.

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* During preparation of this manuscript, Mullertz (*Biochem. J.*, v61, p424, Nov. 1955) reported on similar observations.

Methods. Plasmin, the proteolytic enzyme resulting from the activation of plasminogen, exhibits lysine ethyl esterase activity. Thus, when L-lysine ethyl ester (LEE) is incubated with plasmin, lysine is formed(8). 'Activator' is measured by incubating mixtures of SK and human plasminogen with excess bovine plasminogen and LEE. The amount of lysine liberated under these conditions serves as a measure of the concentration of bovine plasmin, which, in turn, is related to the concentration of activator. The reagents are prepared as follows: the SK, human plasminogen and bovine plasminogen are prepared in pH 6.4 0.1 M phosphate buffer, the SK and human plasminogen at concentrations indicated in the text, the bovine plasminogen, at 4%. A 0.168 M solution of LEE hydrochloride is prepared just prior to use in pH 7.15, 0.1 M phosphate buffer. One ml of each of the 4 reagents is added to the reaction flask; the resulting pH of the reaction mixture is 6.4. Enzyme blanks are prepared by replacing the LEE solution with pH 6.4 buffer, and a substrate blank, by replacing the mixture of SK, human plasminogen and bovine plasminogen with pH 6.4 buffer. The order of addition of reagents is described under each experiment. In certain experiments one or more of the protein components were omitted from the reaction mixture and their blanks in order to establish adequate controls. These are described in each experiment. The tubes are incubated at 37° for 60 minutes. Aliquots are then removed and the lysine determined by a modification(9) of the Spies and Chambers method(10). The results are reported as % hydrolysis of LEE.

TABLE I. Effect of Streptokinase Concentration on Activation of Bovine Plasminogen.

Streptokinase in reaction mixture, units	% hydrolysis of LEE Without bovine plasminogen	With bovine plasminogen
25,000	3.5	45.5
12,500	3.0	41.5
6,250	2.0	31.0
3,125	1.0	24.5

Results. 1) *Effect of streptokinase concentration on ability of a mixture of streptokinase and human plasminogen to activate bovine plasminogen.* Reaction mixtures with and without bovine plasminogen and containing 0.06 mg N of human plasminogen #1, LEE and varying amounts of SK were prepared by mixing the reagents in the above order. The results (Table I) show that with a constant amount of human plasminogen the ability to activate bovine plasminogen increases with increasing SK concentration.

2) *Effect of human plasminogen concentration on ability of a mixture of streptokinase and human plasminogen to activate bovine plasminogen.* This experiment was carried out with the same reagents and order of addition as used in Exp. 1. In this case, however, the SK concentration was kept constant at 6,250 units while the concentration of human plasminogen was varied. The results (Table II) demonstrate that the ability to activate bovine plasminogen is also dependent on the

TABLE II. Effect of Human Plasminogen Concentration on Activation of Bovine Plasminogen.

Human plasminogen reaction mixture, mg	% hydrolysis of LEE Without bovine plasminogen	With bovine plasminogen
.10	2.0	47.0
.08	2.0	31.5
.06	2.0	31.0
.04	.0	22.5
.02	.0	6.0

[†] Unpublished data.

TABLE III.* Effect of Preincubation Time on Ability of a Mixture of Streptokinase and Human Plasminogen to Activate Bovine Plasminogen.

Pre-incubation mixture	Time added, min.	Added to	% hydrolysis of LEE
(a) SK + human plasminogen	2	Bovine plasminogen and LEE	56
	30		39
	45		35
	60		31.5
	90		22

* Conditions described in text.

concentration of human plasminogen.

3) *Effect of preincubation time on ability of a mixture of streptokinase and human plasminogen to activate bovine plasminogen.* A mixture containing 1000 units of SK and 0.35 mg N of human plasminogen #3/ml was incubated at 37°. For assay, 1 ml aliquots were removed at intervals and added to freshly prepared solutions containing bovine plasminogen, LEE and buffer. The data are shown in Table III.

A gradual loss of ability of the mixture of SK and human plasminogen to activate bovine plasminogen is evident; SK and human plasminogen incubated separately were stable.

4) *Effect of addition of streptokinase or human plasminogen on a partially inactivated mixture of streptokinase and human plasminogen.* A mixture containing 200 units of SK and 0.6 mg N of human plasminogen #2 per ml was incubated at 37° and at the stated intervals samples were removed for assay. When partial inactivation had occurred (60 min.—see Table IV), 1 ml aliquots were removed from the solution. The first was used as a control; 0.6 mg N of human plasminogen #2 was added to the second; 200 and 1000 units of SK were added to the third and fourth, respectively. Each aliquot was then assayed for 'activator.' The results are tabulated in Table IV.

TABLE IV.* Effect of Streptokinase and Human Plasminogen on a Partially Inactivated Mixture.

Pre-incubation mixture	Time added, min.	Added to	% hydrolysis of LEE
SK + human plasminogen	2	Bovine plasminogen and LEE	61
	30		53.5
	45		49.5
	60		42
(1) Control	—	"	42
(2) Human plasminogen (.6 mg N)	2		47
(3) 200 SK	2		54
(4) 1000 SK	2		61

Component added to 60 min. preincubation mixture.

* Conditions described in text.

The results indicate that although some increase in the concentration of activator may have occurred on addition of human plasminogen (see also Table II), a greater increase was observed when SK was added, suggesting that the loss of SK may be the cause of the decreased ability of the mixture to activate bovine plasminogen.

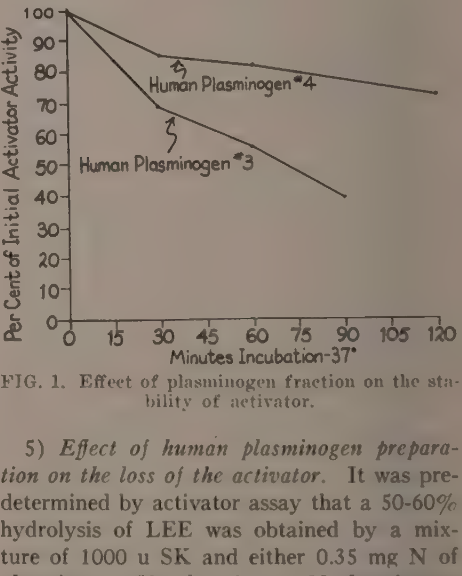

FIG. 1. Effect of plasminogen fraction on the stability of activator.

5) *Effect of human plasminogen preparation on the loss of the activator.* It was predetermined by activator assay that a 50-60% hydrolysis of LEE was obtained by a mixture of 1000 u SK and either 0.35 mg N of plasminogen #3/ml or 0.1 mg N plasminogen #4/ml. These mixtures were incubated at 37° and tested periodically for activator content. The rate of inactivation (Fig. 1) is shown to vary with the human plasminogen fraction employed.

6) *Effect of SK concentration on the loss of activator at pH 7.2.* Since the rate of inactivation could have physiological significance, it was decided to study the effect of SK concentration on the rate of disappearance of the 'activator' at physiological pH. The re-

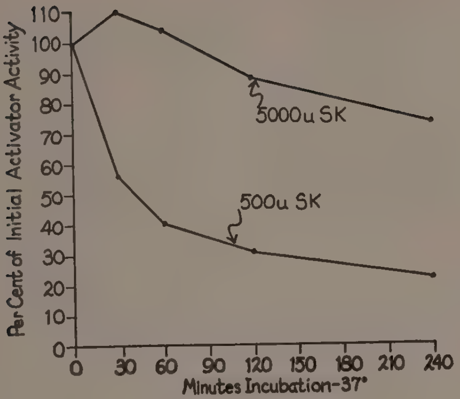

FIG. 2. Effect of SK concentration on stability of activator.

sults were essentially the same as those obtained at pH 6.4. The results (Fig. 2) show that with a constant amount of human plasminogen the rate of decrease of activator is greater at the lower SK concentration.

Discussion. The data presented are interpreted to indicate that SK may be consumed by a "deterioration route" as well as by the route in which it activates plasminogen. It can not be established from these experiments whether the deterioration of activator in a mixture of SK and human plasminogen occurs due to the loss of SK or to the loss of the formed activator, or both. Since variations in SK concentration as well as plasminogen concentration can affect not only the quantity of activator formed but also the rate at which deterioration of activator occurs, the relative rates at which these reactions occur present an obstacle to the study of the kinetics of the activation of plasminogen by SK.

Some speculation on the effects of *in vivo* administration of SK appears to be in order. If SK undergoes reactions *in vivo* as are described herein *in vitro*, its administration to humans(11) could result in variable activation of plasminogen depending on the mode and rate of administration of a given dose of SK. The *in vivo* activation of animal plasminogen by a mixture of SK and human plasma could depend on not only the total dose but also on such factors as the time of administration after mixing, the amount of SK and human plasminogen used, and the deterioration characteristics of the SK-plasminogen mixture.

It should be evident that the fibrinolytic activity appearing in an animal after administration of a mixture of SK and human plasminogen would reflect not only the human plasmin administered but also the activated plasminogen of the animal.

Summary. The ability of a mixture of SK and human plasminogen to activate bovine plasminogen was shown to be dependent upon the concentrations of both components. The 'activator' which was formed, may deteriorate on incubation even though the SK and human plasminogen when incubated separately were stable. The decrease was partially or completely restored by the addition of SK. The rate of disappearance of the 'activator' was affected by the plasminogen preparation and by the initial SK concentration. The inactivation occurred at physiological pH as well as at pH 6.4. The *in vitro* and *in vivo* significance of the data were discussed.

We are grateful to Mr. John Poletto for the LEE preparation, Mr. Merle Querry for some plasminogen fractions, to Mrs. Emma Zilko for carrying out certain assays and to Drs. Edward DeRenzo and Samuel Gordon for their helpful suggestions in preparing this manuscript.

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1. Mullertz, S., and Lassen, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 64.
2. Troll, W., and Sherry, S., *J. Biol. Chem.*, 1955, v213, 881.
3. Sherry, S., *J. Clin. Invest.*, 1954, v33, 1054.
4. Milstone, J. H., *J. Immunology*, 1941, v42, 100.
5. Werbin, H., and Palm, A., *J. Am. Chem. Soc.*, 1951, v73, 1382.
6. Christensen, L. R., and Smith, D. H., Jr., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 840.
7. Oncley, J. L., *et al.*, *J. Am. Chem. Soc.*, 1949, v71, 541.
8. Troll, W., Sherry, S., and Wachman, J., *J. Biol. Chem.*, 1954, v208, 85.
9. Hagan, J., Ablondi, F., and Hutchings, B., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 627.
10. Spies, J., and Chambers, D., *J. Biol. Chem.*, 1951, v191, 787.
11. Tillett, W. S., Johnson, A. J., and McCarty, W. Ross, *J. Clin. Invest.*, 1955, v34, 169.

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Studies on Phenylketonuria. V. Observations on a Newborn Infant with Phenylketonuria.* (22775)

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(Introduced by Emil L. Smith)

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Phenylketonuria, an inherited biochemical abnormality in the oxidation of phenylalanine to tyrosine, is usually associated with mental deficiency(1). Some of the pathological symptoms may be due to the presence of some toxic substance formed as a consequence of the greatly increased amount of phenylalanine which accumulates in patients(2-5). It was important to determine whether newborn infants with phenylketonuria are biochemically normal, since the rate of development of biochemical abnormalities might relate both to the age of onset of symptoms and to the severity of the mental defect observed in later life. There have been indications(5,6) that the urine of newborn infants later found to have phenylketonuria gives a negative reaction for phenylpyruvic acid when tested with ferric chloride. This test is unreliable, however, in establishing the absence of phenylpyruvic acid from dilute samples of urine such as those excreted by small infants, and, in order to determine the degree of biochemical abnormality, it is necessary to use other tests. These are the 2,4-dinitrophenylhydrazine test for phenylpyruvic acid, paper chromatography of an extract of urine in order to detect phenylpyruvic, *o*-hydroxyphenylacetic(7), *p*-hydroxyphenyllactic and indolelactic acids, and determinations of serum levels of phenylalanine. Arrangements were made to obtain blood and urine from infants born to parents who had previously had a child with phenylketonuria. The third infant studied in this manner was phenylketonuric.

Methods. Cord blood was collected immediately after parturition; blood from the newborn infant was collected within 3 hours of birth and at intervals throughout the first month. Serum phenylalanine was determined by a modification(4) of the method of Kapeller-Adler. Urine was collected as frequently as was feasible throughout the first month; unfortunately, the infant observed was a girl, it was impractical to hospitalize her, and urine samples were small, though sufficient for these studies. For qualitative test, the dinitrophenylhydrazine reagent was added to an equal volume of urine; a positive test is shown by the appearance of a yellow turbidity at concentrations of phenylpyruvic acid as low as 2 mg %. This test has proved to be far more reliable than the ferric chloride test for phenylpyruvic acid. Urine was extracted by the procedure described earlier(8); relatively larger volumes of solvents than usual were required because of the small amount of urine available, but the final ethyl acetate solution of the organic acids was conc. in a stream of air and the final volume was adjusted by the addition of ethyl acetate. Small amounts of phenylpyruvic acid may be detected on 2-dimensional chromatograms by the development of a brown color with ammoniacal $AgNO_3$(9); larger amounts are evident as a diffuse pink area on chromatograms which have been sprayed with diazotized sulfanilic acid(12) (phenylpyruvic acid, R_F 0.58 in isopropyl alcohol-aqu. $NH_3 - H_2O = 8:1:1$ ($Ipr - NH_3$) and 0.66 in benzene-propionic acid-water $= 2:2:1$ (organic phase) (Bz-prop.). *o*-Hydroxyphenylacetic acid was estimated as described previously(8), indolelactic acid by spraying 2-dimensional chromatograms with *p*-dimethylaminobenzaldehyde (purple color, R_F 0.48, Ipr-NH_3, and 0.38, Bz-prop.) and *p*-hydroxyphenyllactic acid with diazotized sulfanilic acid.

Clinical summary. Baby M was born at

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* This work was supported by research grants from the N.I.H., U. S. Public Health Service.

term of an uneventful pregnancy; birth weight, 5 lb. 12½ oz. She was completely normal in all respects when followed in the hospital during the newborn period and when examined at the age of one month. She was breast fed and received adequate nourishment. An electroencephalogram, made at the age of 33 days showed evidences of abnormalities. These included isolated spikes and some spike and slow wave complexes of irregular contour, observed in both the waking and sleep records. The record was suggestive of a diffuse cortical disturbance, which appeared to be more marked over the right hemisphere. In order to avoid possible damage to the child, the studies were not continued after phenylpyruvic acid appeared in her urine. She was brought into the hospital at the age of 33 days, and at the age of 40 days she was started on a phenylalanine-deficient regimen.†

Results. The results of the serum phenylalanine determination are shown in Fig. 1; for comparison, values are included from the 2 infants studied previously who proved to be normal. The slow gradual rise of the phenylalanine level in Baby M is in contrast to the fairly rapid (1 day) rise to high levels in older patients with phenylketonuria who were maintained at normal blood phenylalanine levels by a phenylalanine-restricted regimen and then given a natural diet(4). The chromatographic study of the excretion of the minor abnormal metabolites, *o*-hydroxyphenylacetic, indolelactic, and *p*-hydroxyphenyllactic acids showed that, at 5 days of age and with a phenylalanine level of 24 mg %, the infant excreted *none* of the abnormal metabolites always observed in older phenylketonurics. The next urine sample (16 days of age, serum phenylalanine, 47 mg %), contained greatly increased amounts of the above acids, although the rate of excretion of *o*-hydroxyphenylacetic acid was only one-fifth of that observed in older patients with a comparable serum phenylalanine level. The rate of excretion of *o*-hydroxyphenylacetic acid then remained at about one-fifth that observed in older patients.

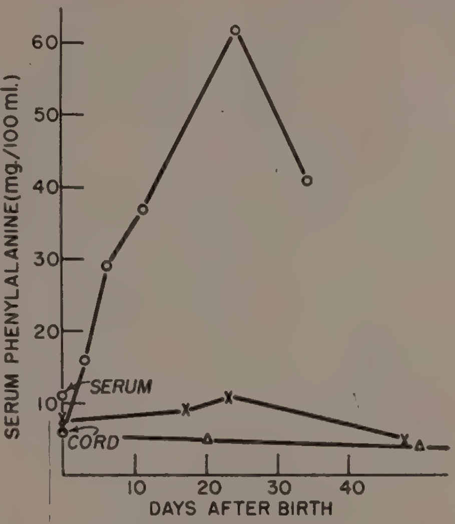

FIG. 1. Serum phenylalanine levels in newborn infant with phenylketonuria. ○, Baby M. Serum sample drawn just after birth was badly hemolyzed. A higher value was obtained than with cord blood, probably because of interference with the normal chromogen. ×, Baby U; △, Baby L.

The most striking finding was that the baby did not excrete a detectable amount of phenylpyruvic acid until it was 34 days old. At this time the urine contained no more than 0.2 mg phenylpyruvic acid per mg creatinine and the daily excretion could have been no more than 7 mg. The excretion of phenylpyruvic acid by most patients is 1.5-3.0 mg/mg creatinine. Phenylpyruvic acid also was clearly detectable for the first time on a 2-dimensional chromatogram of the organic acids extracted from this sample of urine.

Discussion. The possible absence of phenylpyruvic acid from the urine of phenylketonuric infants before the age of one month calls for caution in arriving at a negative diagnosis based on testing during the newborn period the urine of siblings of older children with phenylketonuria. A determination of serum phenylalanine would provide a more reliable test. Although the present report is of a single case, the delay in the development of typical biochemical abnormalities is consistent with previous observations that some phenylketonuric children may develop normally for a few months before developing pathological symptoms(4,5). It might be expected that further observation on other newborns with phenylketonuria will reveal considerable differences in the time required for phenylpyruvic acid to appear in their urine.

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† The diet was similar to that reported previously (4), with the exception that a phenylalanine-deficient casein hydrolysate, supplemented appropriately with amino acids, was used in place of the amino acid mixture.

The delayed appearance of phenylpyruvic acid might be explained most simply by the hypothesis that phenylalanine transaminase is not present in newborn infants with phenylketonuria, and that the production of phenylpyruvic acid and the accompanying fall in serum phenylalanine level indicate the maturation of this enzyme system. A similar development of the tyrosine oxidase system has been shown to occur around the time of birth (10).

Summary. Biochemical studies have been performed on a phenylketonuric infant from birth to the age of 34 days. Cord blood and newborn infant blood contained normal levels of phenylalanine, the serum phenylalanine rose to 62 mg % at 24 days, and decreased to 41% at 34 days. Phenylpyruvic acid was not excreted in detectable amounts until 34 days. *o*-Hydroxyphenylacetic acid was not present in excess at 5 days, but from 16 to 34 days was excreted at about one-fifth the rate usually observed in phenylketonuria. The minor abnormal metabolites indolelactic and *p*-hydroxyphenyllactic acids were not present at 5 days, but were present at 16 days.

We gratefully acknowledge the help of Dr. Stanley W. Wright, Dept. of Pediatrics, University of California Medical Center, Los Angeles, and of Drs. James F. Bosma and Niels L. Low, Dept. of Pediatrics, Univ. of Utah College of Medicine, who obtained materials from the 2 normal infants reported in this study. Eleanor Bethsold carried out the analytical work. We are indebted to Drs. F. A. Horner and C. W. Streamer, Dept. of Pediatrics, Univ. of Colorado Medical Center for their interest and aid in furthering this work. Dr. Lynwood M. Hopple, Children's Hospital, Denver, Colorado, evaluated the E E G. Merck Sharp and Dohme kindly supplied the phenylalanine-deficient basal mix which was given the infant.

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1. Fölling, A., *Z. f. physiol. Chem.*, 1934, v227, 133.
2. Bickel, H., Gerrard, J., and Hickmans, E. M., *Acta pediat.*, 1954, v43, 64.
3. Woolf, L. I., Griffiths, R., and Moncrieff, A., *Brit. Med. J.*, 1955, 1:57.
4. Armstrong, M. D., and Tyler, F. H., *J. Clin. Invest.*, 1955, v34, 565.
5. Horner, F. A., and Streamer, C. W., *J. Am. Med. Assn.*, 1956, v161, 1628.
6. Delay, J., Pichot, P., Delbarre, F., and Taseel, J., *Bull. mem. soc. méd. Hôp. Paris*, 1948, v64, 669.
7. Armstrong, M. D., Shaw, K. N. F., and Robinson, K. S., *J. Biol. Chem.*, 1955, v213, 797.
8. Armstrong, M. D., Shaw, K. N. F., and Wall, P. E., *J. Biol. Chem.*, 1956, v218, 293.
9. Berry, H. K., Sutton, H. E., Cain, L., and Berry, J. S., *Univ. Texas Pub. No.* 5109, 1951, 22.
10. Kretchmer, N., McNamara, H., Barnett, H. L., and Levine, S. Z., *Am. J. Dis. Child.*, 1955, v90, 576.

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Glyoxal and Related Compounds as Potential Blood Sterilizing Agents.
(22776)

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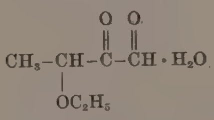
A major problem in the use of human blood and plasma for transfusions is the danger of transmitting the virus of serum hepatitis. No completely satisfactory method is available for inactivation of the virus. Ultraviolet irradiation(1), β-propiolactone (BPL)(2) and a combination of these(3,4) have been studied. Since preliminary studies in our laboratory indicated that glyoxal and many related compounds were potent virucidal agents, experiments were undertaken to determine the potential value of these compounds as blood sterilizing agents by comparing their *in vitro* virucidal activity and their toxicities with other known virucidal agents. Two of these compounds, glyoxal and methyl glyoxal, were previously tested but apparently discarded because when "added to human blood plasma, they caused an increase in the electrophoretic mobilities at pH 8.6 of the plasma proteins."

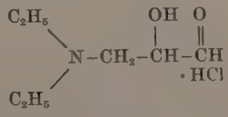
Materials and methods. Viruses: (a) influenza A, PR-8 strain, supplied by Dr. Thomas Francis, Jr., University of Michigan. It was an egg-adapted strain, lethal for 10-day embryos within 60-80 hrs; it reached a titer in 10-day eggs of about 10^9 LD_{50} per 0.1 ml when injected into the allantoic cavity. (b) Newcastle disease virus, NJ-KD strain, secured from Dr. E. S. Pomeroy, University of Minnesota. It was an egg-adapted strain, lethal for 10-day embryos within 50-70 hrs; it reached a titer in 10-day eggs of about 10^8 LD_{50} per 0.1 ml when injected into the allantoic cavity. (c) Mouse hepatitis virus, received from Dr. J. B. Nelson(5). A stock was prepared by injecting 0.1 ml volumes of a 10^{-2} dilution of the virus by the intraperitoneal route into a group of Princeton weanling mice (Millerton Research Farm, Millerton, N. Y.). Fifty hours after infection, the livers of the mice were harvested and a 10% suspension in beef infusion broth was prepared. This material had a titer of approximately 10^7 LD_{50} per 0.1 ml in Princeton weanling mice when injected intraperitoneally.

Chemicals:* (a) *Glyoxal*—A specially purified aqueous solution was obtained from Carbide and Carbon, Inc. The colorless solution was passed through Amberlite IR-45 resin to remove any acid present. The glyoxal content of the resulting solution was 36.8% by weight. (b) Pyruvaldehyde (methyl glyoxal)—This was obtained from the same source and purified in the same way as the glyoxal. The methyl glyoxal content of the aqueous solution was 33.4% by weight. (c) Formaldehyde—A fresh commercial sample (37% aqueous solution) was filtered through paper and used directly. (d) β-propiolactone—A redistilled commercial sample (B. F. Goodrich Co.) was used. To minimize hydrolysis, the BPL dilutions were made immediately before using. Water was used as the solvent except when the lysis of erythrocytes was being studied; then saline was used. (e) Kethoxal (β-ethoxy-α-ketobutyraldehyde hydrate); U-1671 (β-diethylaminolactaldehyde hydrochloride); and Kethoxal bisulfite—A description of the synthesis of these compounds will be published elsewhere. The structures of Kethoxal and U-1671 are shown below:



$$CH_3{-}\underset{OC_2H_5}{\underset{|}{CH}}{-}\overset{O}{\overset{\|}{C}}{-}\overset{O}{\overset{\|}{CH}} \cdot H_2O$$

Kethoxal



U-1671 (Upjohn code No.)

Test for virucidal activity. One of 3 media was used: (a) A mixture of equal volumes of beef infusion broth and physiological saline, (b) whole blood, (c) plasma. Virus was incubated for 2½ hrs at 37°C with equimolar quantities of the various test chemicals. The amount of infective virus remaining in each sample after incubation was determined by making serial 10-fold dilutions in 1:1 beef infusion broth:saline and titrating in groups of 10 eggs or mice, depending on the virus used. Deaths were recorded for a period of 10 days, and the titers were calculated by the method of Reed and Muench(6).

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* The chemical synthesis and purification procedures were conducted by Dr. R. H. Levin and his associates in the Chemistry Department of The Upjohn Co.

Test for Lysis of Erythrocytes. Equimolar quantities of the test chemicals were mixed with 10 ml of 1% human red blood cells suspended in physiological saline. Five tubes for each chemical and 5 control tubes were prepared. The tubes were continuously shaken on a mechanical shaker at 25°C. The amount of lysis was determined at intervals by centrifuging one tube from each set for 5 min at 2000 rpm in a Model V International Centrifuge and determining the optical density of the supernatant at a wavelength of 590 mμ in a Beckman Model B Spectrophotometer. A standard curve was previously drawn in which optical density was plotted against known concentrations (0.1-1.0%) of red blood cells which were completely lysed by placing them in distilled water for 10 min. The % lysis of the test samples was determined by referring optical density measurements to this standard curve. A control tube which had been shaken for the same length of time served as the blank.

Electrophoresis studies. Equimolar amounts of the test chemicals were added to human plasma. After incubation under varying conditions, each sample was diluted with an equal volume of veronal buffer, pH 8.6 and ionic strength 0.1 and dialysed against 250 ml of the same buffer for 24 hrs. The samples were then diluted to approximately 1.5% total protein concentration and dialysed for another 24 hr against 2 L of fresh veronal buffer. The dialysed samples were then subjected to electrophoresis for a period of 3 hr.[†]

TABLE I. Comparative Virucidal Activities of Various Chemicals vs. Influenza A (PR-8) and Newcastle Disease (NJ-KD) Viruses.

Equimolar quantities of the test chemicals, each in a vol of 0.1 ml, were incubated with 0.1 ml of 10-fold diluted (Exp. 1) or non-diluted (Exp. 2 and 3) infective allantoic fluid in 4.8 ml of the indicated medium. Each sample was incubated for 2½ hr at 37°C, and the amount of residual infective virus was then determined by making serial 10-fold dilutions and titrating in groups of 10 eggs. Each dilution was inj. intra-allantoically at a level of 0.5 ml/egg. Results are expressed as the $\log_{10}$ titer of 1 LD_{50}.

Exp. No.*	Chemical	Molar conc.	Final titer
1 a	BPL	1.35×10^{-4}	8.7
	Formaldehyde	"	8.9
	Kethoxal	"	6.5
	U-1671	"	8.4
	Control	—	9.0
1 b	BPL	1.35×10^{-4}	6.9
	Formaldehyde	"	7.4
	Kethoxal	"	5.6
	U-1671	"	7.0
	Control	—	7.5
2	BPL	1.35×10^{-4}	8.7
	Formaldehyde	"	8.9
	Kethoxal	"	7.5
	U-1671	"	8.5
	Control	—	9.1
3 a	Kethoxal	2.70×10^{-3}	6.7
	"	4.05×10^{-3}	5.1
	"	5.40×10^{-3}	2.8
	"	6.75×10^{-3}	<2.0
	BPL	"	6.0
	Formaldehyde	"	5.3
	U-1671	"	8.1
	Kethoxal bisulfite	"	6.2
	Glyoxal	"	<2.0
	Methyl glyoxal	"	7.4
	Control	—	8.1
3 b	Kethoxal	2.70×10^{-3}	5.1
	Glyoxal	"	4.2
	Control	—	6.4

* The medium used in Exp. 1 was 50% beef broth, in Exp. 2 was chicken plasma, and in Exp. 3 was chicken blood. The PR-8 virus was used in Exp. 1 a, 2 and 3 a; NJ-KD was used in Exp. 1 b and 3 b. The initial titer of the viruses in each exp. was as follows: 1 a = 9.4; 1 b = 7.9; 2 = 9.2; 3 a = 9.3; 3 b = 7.6.

Results. Comparative in vitro virucidal activity of various chemicals. Comparative virucidal activities of several of the test chemicals were determined (Table I). Kethoxal was a more potent virucidal agent in broth, plasma or whole blood medium than were equimolar amounts of BPL, formaldehyde or U-1671. Experiment 3 (Table I) further indicated that Kethoxal and glyoxal were more potent virucidal agents for PR-8 virus in whole blood medium than were any of the other chemicals tested; that a molar concentration of approximately 6.75×10^{-3}

[†] The electrophoresis determinations were conducted by G. C. Colovos of The Upjohn Company, Physics Department.

TABLE II. Comparative Virucidal Activities vs. Mouse Hepatitis Virus.

Equimolar quantities of the test chemicals, each in a vol of 0.1 ml, were incubated with 0.5 ml of a 10% liver suspension of mouse hepatitis virus in 4.4 ml of whole chicken blood. Each sample was incubated for 2½ hr at 37°C, and the amount of residual infective virus was then determined by making serial 10-fold dilutions and titrating in Princeton weanling mice. Each dilution was inj. intraper. at a level of 0.1 ml/mouse. Deaths were recorded for a period of 10 days. Results are expressed as the reciprocal of the $\log_{10}$ titer of 1 LD_{50}.

Exp. No.	Chemical	Molar conc.	Final titer
1	Kethoxal	6.75×10^{-3}	2.1
	BPL	"	3.4
	Glyoxal	"	<2.0
	Control	—	6.8
2	Kethoxal	3.38×10^{-3}	4.0
	BPL	"	6.1
	Glyoxal	"	3.6
	Control	—	7.0

(1000 mg/L) of Kethoxal was required to inactivate all PR-8 virus in whole blood under the stated incubation conditions; and that glyoxal was slightly more potent than Kethoxal against NJ-KD virus in whole blood. The comparative virucidal activities of Kethoxal, BPL and glyoxal against the virus of mouse hepatitis were studied. This virus was chosen because of its possible closer relationship to the virus of serum hepatitis. The results (Table II) indicated that Kethoxal and glyoxal were more active than BPL against the virus of mouse hepatitis.

Comparative effect on lysis of erythrocytes. An experiment was designed to test the comparative effects of Kethoxal, BPL and glyoxal on a 1% suspension of human red blood cells. All 3 chemicals were used at a molar concentration of 6.75×10^{-3}. The results (Table III) indicated that BPL lysed the red cells more rapidly than did Kethoxal or glyoxal. When whole blood was used, the results were different. Equimolar amounts of the chemicals were mixed with whole human blood and stored for 3 weeks at 4°C, similar to storage conditions used in hospital blood banks. Under these conditions, it was found that a molar concentration of 4.05×10^{-2} (6000 mg/L) of Kethoxal caused 2.3% lysis of the erythrocytes of whole blood, an equimolar amount of BPL caused 2.0% lysis, and an equimolar amount of glyoxal caused 1.4% lysis. A control sample of human blood stored for the same period of time was found to have undergone 1.1% lysis of the red cells, indicating that the net lytic effect of the glyoxal (0.3%) was very low. Spectrophotometer readings on the supernatants from these whole blood samples were made at a wavelength of 490 mμ. The lower wavelength was used with the whole blood samples because at 490 mμ there was less interference from the yellow color apparently formed by reaction of the carbonyl compounds with blood proteins. No attempt was made in these experiments to correct the values for possible formation of methemoglobin.

TABLE III. Comparative Effect of Equimolar Amounts of Kethoxal, β-Propiolactone and Glyoxal on Lysis of a 1% Suspension of Human Red Blood Cells.

Kethoxal was used at a molar conc. of 6.75×10^{-3} (1000 mg/L). The figures in the table express % of lysis.

Time after mixing (hr)	Kethoxal	β-Propiolactone	Glyoxal
1	0	41	0
2	0	57	0
4	0	85	0
6	23	92	0
7	31	95	0

Comparative effect on plasma proteins. In order to determine the effect of Kethoxal on plasma proteins, a fresh sample of human plasma was obtained and treated with Kethoxal at a molar concentration of 6.75×10^{-3} (1000 mg/L); this was the minimum concentration previously found to destroy all detectable Newcastle and influenza viruses in artificially infected whole blood. A second portion of the plasma was treated with an equimolar amount of glyoxal, a third portion with an equimolar amount of BPL, and a fourth portion served as a control. Each sample was incubated for 93 hr at room temperature, then for an additional 48-72 hr at 4°C. In a similar second experiment, one group of samples received the 3 chemicals at a molar concentration of 6.75×10^{-3}, as above; another group of plasma samples received double this amount of each chemical.

This group of 8 samples, 6 treated and 2 controls, was incubated for 21 days at 4°C, then subjected to electrophoresis. It was found that the presence of the various chemicals under the stated incubation conditions caused no significant changes in the electrophoretic pattern of the human plasma.

Discussion. The results of these experiments indicate that glyoxal and Kethoxal may have value as virucidal agents. Both chemicals were more potent in destroying the infectivity of influenza A, Newcastle disease and mouse hepatitis viruses than was an equimolar amount of BPL. Glyoxal appeared to be less lytic than BPL or Kethoxal, and none of the chemicals caused significant changes in human plasma proteins at concentrations which were effective in sterilizing artificially-infected blood. These results suggest that glyoxal and Kethoxal hold some promise as blood-sterilizing agents. Glyoxal gave results as good or better than Kethoxal. Since thorough studies have not been made on the toxicity of glyoxal for mammalian hosts(7,8), further work is required before it can be applied to the large-scale sterilization of blood and plasma. The marked superiority of glyoxal over formaldehyde as a virus inactivating agent suggests also the possible application of glyoxal in the preparation of "killed" vaccines or as a general viral disinfectant.

Summary. Both glyoxal and β-ethoxy-α-ketobutyraldehyde (Kethoxal) were more potent virucidal agents than β-propiolactone (BPL). Glyoxal was less lytic for human erythrocytes than was Kethoxal or BPL. None of the chemicals showed significant toxicity for plasma proteins as measured by electrophoresis. These experiments indicate that glyoxal may be of potential value in the sterilization of human blood and plasma.

1. Murray, R., Oliphant, J. W., Tripp, J. T., Hampil, B., Ratner, F., Diefenbach, W. C. L., and Geller, H., *J. Am. Med. Assn.*, 1955, v157, 8.

2. Hartman, F. W., LoGrippo, G. A., and Kelly, A R., *Am. J. Clin. Path.*, 1954, v24, 339.

3. Smolens, S., and Stokes, J., Jr., Proc. Soc. Exp. Biol. and Med., 1954, v86, 538.

4. Hartman, F. W., Kelly, A. R., LoGrippo, G. A., *Gastroenterology*, 1955, v28, 244.

5. Nelson, J. B., *J. Exp. Med.*, 1952, v96, 293, 303.

6. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

7. Sakuma, F., *J. Biochem. (Japan)*, 1931, v13, 423.

8. Doerr, W., Bopp, F., Kuhn, R., and Quadbeck, G., *Naturwiss.*, 1948, v35, 125.

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Increased Erythropoietic Stimulant in Plasma of Pregnant Rats.[*] (22777)

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The existence of an erythropoietic substance in the blood stream was first suggested by Carnot and Deflandre(1) who showed that plasma from rabbits which had been bled repeatedly when injected into other rabbits resulted in hyperplastic bone marrow and increased reticulocyte and erythrocyte counts. Several investigators have since(2-19) confirmed the presence of erythropoietic activity in plasma of animals which had been bled. Plasma from animals exposed to low oxygen tensions has similarly been found to stimulate erythropoiesis in recipients (6b,9,20-22, 24b). Human umbilical cord blood, also fetal sheep and dog blood, have been found to increase red blood cell count in recipient animals(22-26). As these reports of an erythropoietic substance in blood were based either on reticulocyte counts, red blood cell counts, or hemoglobin determinations, and as these methods are subject to error introduced by changes in plasma volume, it is not surprising that other investigators have been unable to confirm the presence of an erythropoietic substance in blood(27-29).

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It was the object of this study to reinvestigate the existence of an erythropoietic stimulant in the blood stream of the rat, using a more satisfactory method for determination of increased numbers of red cells in the circulation, namely, the total circulating red cell volume. Changes which may exist under differing physiological conditions, such as pregnancy and fetal life, would then be on a more sound basis. This paper compares the levels of erythropoietic factor in plasma of pregnant and normal female rats.

Material and methods. The test animals for measuring red cell volume were female rats of the Long-Evans strain, hypophysectomized on the 26th to the 28th day of life and maintained for 45 to 50 days to allow stabilization of the hematological values at the low level characteristic of hypophysectomized rats. The anemia which develops within this period in such animals is severe, the red cell volume being 50% below normal. This anemia is known to be promptly repaired after administration of erythropoietic stimulants such as cobalt, plasma factor and pituitary extracts(15,30-32). The plasma from pregnant rats was newly prepared for each day's injection, as follows: Between the 15th and 18th days of pregnancy the donors were exsanguinated by withdrawal of blood from the aorta into a heparinized syringe. The blood was transferred at once to centrifuge tubes containing a small amount of mineral oil to exclude air, and was centrifuged for 12 minutes at 2,000 rpm. The plasma was drawn into a syringe and immediately injected intraperitoneally. This procedure was repeated daily for 14 days. Groups consisting of 6 to 8 hypophysectomized rats received daily doses of 2 or 4.8 cc. Other groups received 2 or 4.8 cc of plasma similarly prepared from normal adult female rats of the same age as the pregnant donors. At the termination of the ex-

TABLE I. Increased Hematological Values of Hypophysectomized ($\overline{\text{H}}$) Rats Injected with Plasma from Normal (N) or Pregnant Rats.

Type of plasma	Daily dose 14 d, ml	No. rats	Body wt, g*	Hemoglobin, g: Per 100 ml	Hemoglobin, g: Total	Hemoglobin, g: Per 100 g body wt	Blood vol per 100 g body wt, ml	Plasma vol per 100 g body wt, ml	Hematocrit, %	Red cell vol, ml: Total	Red cell vol, ml: Per 100 g body wt, ml
Pregnant	2	26	82 ± 1	9.3 ± .2	.42 ± .02	.51 ± .01	5.52 ± .10	3.85 ± .20	30.4 ± .2	1.38 ± .12	1.68 ± .05
	4.8	7	84 ± 1	10.3 ± .1	.56 ± .01	.67 ± .02	6.44 ± .15	4.45 ± .10	30.8 ± .1	1.67 ± .09	1.99 ± .03
Normal	2	22	85 ± 1	9.2 ± .2	.40 ± .01	.47 ± .02	5.18 ± .18	3.73 ± .08	28.5 ± .1	1.23 ± .11	1.45 ± .02
	4.8	6	81 ± 1	10.0 ± .2	.47 ± .01	.58 ± .01	5.82 ± .15	4.04 ± .10	30.5 ± .1	1.44 ± .11	1.78 ± .02
Controls											
$\overline{\text{H}}$		19	76 ± 1	9.3 ± .2	.34 ± .02	.45 ± .01	4.80 ± .07	3.41 ± .09	28.9 ± .1	1.06 ± .07	1.39 ± .04
N		29	198 ± 3	13.0 ± .2	1.32 ± .02	.67 ± .02	5.13 ± .08	2.88 ± .08	43.9 ± .5	4.46 ± .09	2.25 ± .03

* Stand. error $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

periment control hematological values were determined in uninjected hypophysectomized and normal rats of the same age as the experimental groups. Red cell volumes of all rats were determined by the Fe^{59} tagged cell method(31). The result obtained from injection of the lower dose level (2 cc of plasma) was confirmed 3 times. All hypophysectomized rats were fed a standard laboratory diet (Diet XIV) consisting of: wheat 68.5%, casein 5%, fish meal 10%, alfalfa leaf meal 10%, fish oil 5%, NaCl 1.5%, KI added (analysis 1 μg iodine per g diet). Each afternoon a dish of liquid diet was given consisting of Diet I (modified McCollum Diet I): wheat 67.5%, casein 15%, skim milk powder 7.5%, fish oil 1%, NaCl 0.75%, $CaCO_3$ 1.5%, KI added (analysis 1 μg iodine per g diet). Lettuce was fed twice weekly. Pregnant and lactating rats were maintained on Diet I.

Results. The results are presented in Table I. The hypophysectomized rats injected with the daily dose of 2 cc of plasma from pregnant rats showed a significant increase in total circulating red cell volume (21%), also a significant increase in red cell volume per 100 g of body weight ($P = < .001$). The increase in circulating red cell volume was accompanied by an increase in total circulating hemoglobin of 14%, and of 13% in hemoglobin per 100 g of body weight ($P = < 0.01$). Injection of the larger dose of plasma from pregnant rats (4.8 cc) increased total circulating hemoglobin by 51%, the hemoglobin per 100 g of body weight by 40%, circulating red cell volume and red cell volume per 100 g body weight by 43% ($P = < .001$). No increase in hematological values occurred in hypophysectomized rats injected with the 2 cc dose of plasma from normal rats. At the higher daily dose of normal plasma (4.8 cc) which was equivalent to, or greater than, the recipient's total blood volume, an increase of 23% in red cell volume per 100 g body weight ($P = < .001$), and of 21% in hemoglobin per 100 g body weight resulted ($P = < .001$). The stimulation to erythropoiesis, when normal plasma was used, was definitely less than that observed from injection of plasma from pregnant rats ($P = < .001$).

The hematocrit and hemoglobin/100 ml were not increased in any injected hypophysectomized rats as there was a concomitant increase in plasma volume and in total blood volume. Increase in plasma volume was more pronounced in those injected with plasma from pregnant rats, ranging from 13% ($P = < .001$) at the lower dose level to 34% ($P = < .001$) at the higher level. The increase in plasma volume in rats injected with normal serum was only 8% ($P = < .001$) at the lower dose level, increasing to 19% ($P = < .001$) at the higher level. The increased plasma volume probably is attributable to the growth promoting substances (or growth hormone) which has been shown to be present when this amount of plasma from normal or pregnant rats was given. Pituitary growth hormone has been shown to increase plasma volume(34).

Increase in red cell volume and hemoglobin in hypophysectomized rats injected with plasma from normal rats is further evidence that a circulating erythropoietic factor is normally present in the blood stream, and the higher values in rats injected with plasma from pregnant rats indicates that increased amounts of erythropoietic substance circulate during pregnancy. Increased production during pregnancy of this factor, or factors, may be responsible for the adult level of the red cell volume which characterizes the rat at birth; withdrawal of this stimulus at birth may account for the physiological anemia developing in the neonatal period(33). If this is true, then plasma from pregnant rats should prevent the neonatal anemia.

To test this concept newborn rats were injected during the postnatal period with plasma from pregnant rats and the results were compared with those from injection of normal rat plasma. Experimental groups were so constituted that each group consisted of 6 male rats, each from a different litter. Two of the 6 rats received normal rat plasma, 2 received pregnant rat plasma, and 2 were uninjected controls. (The newborn rats were identified by clipping toes, or by their color and coat pattern.) Care was taken to maintain the mothers on a diet satisfactory for lactation. The injections were started the 4th

TABLE II. Prevention of Neonatal Anemia by Injections of Pregnant Rat Plasma.

Type of plasma inj.	No. rats	Body wt, g*	Hemoglobin, g			Blood vol, ml	Plasma vol, ml	Hematocrit, %	Red cell vol, ml	
			Per 100 ml	Total	Per 100 g body wt	Per 100 g body wt	Per 100 g body wt		Total	Per 100 g body wt
Pregnant	40	45.0 ± .5	6.8 ± .1	.21 ± .01	.46 ± .01	6.78 ± .10	4.78 ± .08	29.5 ± .3	.90 ± .03	2.00 ± .03
Normal	29	45.2 ± 1.0	6.3 ± .1	.19 ± .01	.41 ± .01	6.52 ± .09	4.89 ± .10	27.6 ± .7	.83 ± .01	1.83 ± .04
No inj.	45	44.1 ± .5	6.7 ± .2	.18 ± .01	.41 ± .01	6.25 ± .10	4.60 ± .07	26.4 ± .3	.73 ± .01	1.66 ± .02

* Stand. error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

day of life and were continued daily for 14 consecutive days or until the rats were 18 days of age at which time the neonatal anemia is known to be most severe(33). The suckling rats were removed from cages only for the short periods required for injection. After injection each group of 6 suckling rats was placed with a different lactating female in order to equalize the milk supply. With proper care the young were not rejected because of this procedure.

The plasma was prepared by the procedure described previously. The dose given was 1 cc per day for the first 5 days, and 2 cc per day for the remainder of the experimental period.

The combined results of 4 repetitions of this experiment are presented in Table II. A statistically significant ($P = < .001$) increase (22%) occurred in the red cell volume of the newborn rats injected with plasma from pregnant rats. A smaller increase, 10% ($P = < .05$), occurred when the newborn rats received plasma from normal female rats. A significant increase also occurred in other hematological values. The anemia of the newborn rat was ameliorated, though not completely prevented, by injection of plasma from pregnant rats; the values were 10% below normal adult levels but were 22% above typical postnatal levels. From the results obtained in newborn rats, as well as in hypophysectomized rats, it may be concluded that the plasma from pregnant rats has an erythropoietic potency greater than that of the plasma from normal adult female rats. These results therefore support the assumption that an erythropoietic principle in pregnant rat serum plays a role in fetal red cell production. The site of the production of this erythropoietic factor is under investigation.

Summary. 1. Plasma of normal adult female rats contains a factor capable of stimulating erythropoiesis in hypophysectomized and newborn rats, as judged by increased circulating red cell volume and hemoglobin. 2. This humoral erythropoietic factor is markedly increased during pregnancy. 3. Injections of plasma from non-pregnant and pregnant rats resulted in an increase in plasma

and blood volumes of hypophysectomized and newborn recipients.

1. Carnot, P., *C. R. Soc. Biol.*, 1906, v61, 463; Carnot, P., and Deflandre, Cl., *C. R. Acad. Sci.*, 1906, v143, 384, 432.

2. Gibelli, C., *Arch. Exp. Path. u. Pharmak.*, 1911, v65, 284.

3. Müller, P. T., *Arch. f. Hyg. u. Bact.*, 1912, v75, 290.

4. Mansfeld, G., *Pflüg. Arch. f. Physiol.*, 1913, v152, 23.

5. Giribaldi, G., *Biochem. e. therap. Sper.*, 1920, v7, 52.

6. Förster, J., and Kiss, F., *Biochem. Ztschr.*, 1925, v160, 442; 1924, v145, 309.

7. Sih, A., *Endokrinologie*, 1928, v1, 83.

8. Krahenbuhl, G., *Pflug. Arch. f. Physiol.*, 1933, v232, 848.

9. Tei, Yu-Tin, *J. Chosen M. A.*, 1938, v28, 1, 173, 179, 185.

10. Ruhenstroth-Bauer, G., *Arch. Exp. Path. u. Pharmak.*, 1950, v211, 32.

11. Krumdiek, N., PROC. SOC. EXP. BIOL. AND MED, 1943, v54, 14.

12. Gunther, B., Hodgson, G., Tohà, J., and Quappe, O., *Acta Physiol. Latino-Am.*, 1951, v1, 271; Hodgson, G., Tohà, J., and Gonzalez, E., *Bol. Soc. Biol. Conception*, 1952, v27, 69; *Blood*, 1954, v9, 299.

13. Erslev, A. J., Lavietes, P., and van Wagenen, G., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 548; *Blood*, 1953, v8, 349; 1954, v9, 1055; 1955, v10, 954.

14. Borsook, H., Graybiel, A., Keighley, G., and Windsor, E., *ibid.*, 1954, v9, 734.

15. Gordon, A. S., Piliero, S. J., Kleinberg, W., and Freedman, H. H., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 255; Gordon, A. S., Piliero, S. J., and Tannenbaum, M., *Am. J. Physiol.*, 1955, v181, 585; Gordon, A. S., Piliero, S. J., Tannenbaum, M., and Sieger, C. D., PROC. SOC. EXP. BIOL. AND MED., 1956, v89, 246.

16. Stohlman, F., Jr., and Brecher, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 1.

17. Butzengeiger, K. H., and Lange, J., *Klin. Wchnschr.*, 1952, v30, 647.

18. Gley, P., *Bull. de l'Acad. Nat. de Med.*, 1952, v116, 521; 1954, v138, 435; Gley, P., Delor, J., and Laur, C. M., *C. R. Soc. Biol.*, 1954, v148, 780.

19. Plzak, L. F., Fried, W., Jacobson, L. O., and Bethard, W. F., *J. Lab. Clin. Med.*, 1955, v46, 671; Fried, W., Plzak, L., Jacobson, L. D., and Goldwasser, E., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 203.

20. Reissmann, K. R., *Blood*, 1950, v5, 372.

21. Kinard, F. W., and Ellis, D. W., *Anat. Rec.*, 1949, v105, 556.

22. Westphal, U., *Erg. Physiol.*, 1944, v31-45, 482.

23. Klingelhöffer, K. O., *Pflug. Arch. f. Physiol.*, 1948, v250, 465.

24. Bonsdorff, E., *Acta Physiol. Scandinavica*, 1949, v18, 51; Bonsdorff, E., and Jalavisto, E., *ibid.*, 1948, v16, 150; Bonsdorff, E., *3rd Inter. Congr. Hemat.*, 1950, p82.

25. Döring, G. K., and Loeschcke, H. H., *Pflug. Arch. f. Physiol.*, 1949, v251, 220.

26. Loeschcke, H. H., *Ztschr. f. Vitamin-Hormon und Fermentforschung*, 1949-50, v3, 346; Schwartzer, K., and Loeschcke, E., *Klin. Wchnschr.*, 1940, v19, 64.

27. Leffkowitz, M., and Leffkowitz, A., *Ztschr. f. d. ges. exp. Med.*, 1926, v48, 276.

28. Gordon, A. S., and Dubin, M., *Am. J. Physiol.*, 1934, v107, 704.

29. Feenders, H., *Frankf. Ztschr. f. Path.*, 1936, v49, 41.

30. Crafts, R. C., *Endocrinology*, 1954, v54, 84; Crafts, R. C., and Meineke, H., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 222.

31. Contopoulos, A. N., Van Dyke, D. C., Simpson, M. E., Lawrence, J. H., and Evans, H. M., *Endocrinology*, 1954, v55, 808.

32. Garcia, J. F., Van Dyke, D. C., and Berlin, N. I., PROC. SOC. EXP. BIOL. AND MED., 1952, v80, 472.

33. Contopoulos, A. N., Van Dyke, D. C., Simpson, M. E., Lawrence, J. H., and Evans, H. M., *Blood*, 1955, v10, 115.

34. Batts, A. A., Bennett, L. L., Garcia, J., and Stein, J., *Endocrinology*, 1954, v55, 456.

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Production of Anti-Human PTC and Anti-Human Proconvertin in Rabbits.*
(22778)

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The appearance of circulating anticoagulants has been described in both hemophilia A(1-12) and hemophilia B (PTC deficiency, Christmas Disease)(13,14). Presumably, in both diseases, the inhibitors have arisen as antibodies against "foreign" proteins, either the AHF or the PTC of therapeutically infused normal plasma. In each disease it has been possible to demonstrate that the inhibitor is specific to the factor missing in the particular patient(15). The preparation, in animals, of similar specific antibodies to the various human clotting factors could prove of great value in the study of blood coagulation and, if precipitin reactions occur, in assays of the various factors. Pinniger and Prunty (16) and Hardisty and Pinniger(17) describe the preparation, in rabbits, of an anti-human fibrinogen giving positive precipitin reactions to human plasma and fibrinogen but negative to serum and the plasma of an afibrinogenemic patient. Recently Richards and Spaet (18) have, by immunization of rabbits with human AHF, prepared a rabbit serum highly inhibitory to human AHF, and giving positive precipitin tests to the antigen. However, they do not describe precipitin tests with normal plasma, serum, or hemophilic plasma.

These studies were undertaken in an attempt to prepare rabbit antisera with specific anti-PTC activity. Rabbits injected with our "PTC" preparations formed inhibitors to both PTC and proconvertin. Attempts were made to separate and further purify the antiserum by adsorption with human plasmas from patients congenitally deficient in PTC or proconvertin.

Methods. Preparation of serum PTC. A number of different PTC preparations were made, often with slight modifications of the following: Outdated citrated blood bank plasma was dialysed 48 to 72 hours against 0.01 M sodium oxalate in 0.85% NaCl, recalcified with 1/50 volume 1 M $CaCl_2$, allowed to stand overnight (4°C) and the clot removed. Barium sulfate, 10 mg/ml, was stirred for 10 min. with the serum, centrifuged, washed twice with 0.85% NaCl, twice with distilled water and eluted twice with 1/10 the plasma volume of 0.2 M sodium citrate. The eluates were combined, dialysed 24 hours against water and lyophilized to 1/20 the original plasma volume. The specific resistance was adjusted to that of 0.85% NaCl and the pH to 7.1. Tests on these "PTC" fractions revealed that they contained 5 to 10 times the plasma concentration of PTC, some proconvertin, little prothrombin and no detectable thrombin, AHF, proaccelerin, Hageman factor or fibrinogen. Initial attempts at immunization involved intravenous injections in 3 rabbits, 2 of which convulsed and died within seconds after the fifth injection and showed adherent clots in the ventricles. The remaining 8 rabbits were immunized with intramuscular injections: 1 ml 3 times a week for the first week, 2 ml 3 times a week for the second week, a 10 day rest, followed by a 5 ml injection. Blood was obtained by cardiac puncture 12 and 24 days later. Initial tests, employing rabbit serum, frequently caused clotting when plasma was present in the system. Therefore, in most instances, whole or $BaSO_4$ absorbed oxalated rabbit plasma was used. *Plasma Fractions.* AHF: 0.5 ml rabbit plasma was mixed with 0.1 ml 95% alcohol, chilled 1 hour, the precipitate collected and dissolved in 0.5 ml 0.85% NaCl. *PTC*: 1.5 ml rabbit plasma was mixed with 150 mg $BaSO_4$, centrifuged, the $BaSO_4$ washed once with distilled water,

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drained and eluted with 0.5 ml 0.2 M sodium citrate. This eluate was diluted 1-10 before testing, and contained prothrombin and proconvertin as well as PTC. *Clotting tests.* Most of the methods have been described(19, 20). Hageman factor was measured in a manner similar to AHF, but employing as a substrate plasma from a patient with Hageman factor deficiency(21). This patient was seen through the courtesy of Dr. Oscar Ratnoff. Titers for AHF, PTC, Hageman factor, prothrombin, proconvertin and proaccelerin were carried out on 1-10 dilutions of plasma. Tests for inhibitors to these factors were carried out by incubating 1-5 diluted normal human plasma with equal volumes of normal or injected $BaSO_4$ adsorbed rabbit plasma or saline for 30 minutes and then measuring the residual activity in each test system. When this activity was less than that of the saline-incubated human plasma, inhibition had occurred. Complete inhibition was recorded as 4+. Antifibrinogen was measured by incubating equal volumes of whole human plasma and whole rabbit plasma and, after 30 minutes, measuring fibrinogen concentration of the mixtures turbidometrically(2). This concentration could then be compared to that calculated for the mixtures, the concentration of each component having been measured separately. Anti-thrombin activity was estimated by comparing the clotting times of normal and injected rabbit plasma after addition of human thrombin. Anti-thromboplastin was measured by comparing clotting times of normal and injected rabbit plasmas, mixed with human brain suspension for 30 minutes and recalcified. Anti-human plasma thromboplastin was measured by allowing a normal human thromboplastin generation (modified from(22)) mixture (0.2 ml $BaSO_4$ plasma (1-5) + 0.2 ml serum (1-10) + 0.2 ml platelet suspension + 0.2 ml 0.025 M $CaCl_2$) to incubate 6 minutes, removing 0.1 ml samples, incubating these with 0.1 ml of control or injected rabbit $BaSO_4$ adsorbed plasma or with 0.85% NaCl for 5 minutes and recording the clotting times on addition of 0.1 ml human citrated plasma and 0.1 ml 0.025 M $CaCl_2$.

TABLE I. Blood Clotting Factors in the Rabbits.

	Normal	Injected
Clotting time (min.)	5	6
Prothrombin consumption, %	95	95
Platelet count (per mm³)	566,000	662,000
Recalcification time (sec.)	120	120
Prothrombin time (sec.)	7.6	7.3
Prothrombin*	85	105
Proconvertin*	190	225
Proaccelerin*	200	300
AHF*		
on plasma	100	20
on fraction	100	120
PTC*		
on plasma	150	20
on fraction	100	100
Hageman Factor*	100	<100
Fibrinogen*	125	117

* As % of normal human level.

Results. Blood coagulation studies in the rabbits. (Table I, Fig. 1). Clotting times, prothrombin consumptions and platelet counts carried out on one rabbit from the injected group and one from the control group did not show any significant differences. The 8 injected rabbit plasmas all showed anti-human PTC, so were pooled and compared to pooled control rabbit plasma in the remaining tests. Hageman factor appeared to be normal and proaccelerin and proconvertin far higher in both normal and injected rabbit plasma than in human plasma. Only assays for AHF and PTC were significantly low in the injected group. As these assays are carried out on human substrates, *i.e.* plasmas from patients with hemophilia A or B, it is

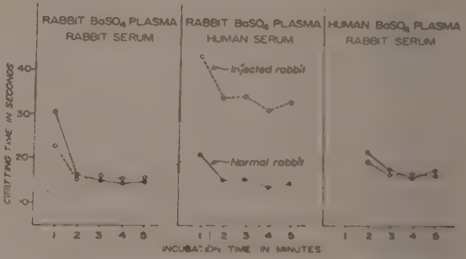

FIG. 1. Thromboplastin generation tests. All tests contained 0.2 ml rabbit platelets plus 0.2 ml 0.025 M $CaCl_2$. Substrate in all was normal human citrated plasma. Part I. 0.2 ml rabbit $BaSO_4$ adsorbed plasma (1-5) plus 0.2 ml rabbit serum (1-10) from normal and inj. rabbits. Part II. 0.2 ml normal or inj. rabbit $BaSO_4$ plasma (1-5) plus 0.2 ml human serum (1-10). Part III. 0.2 ml human $BaSO_4$ plasma (1-5) plus 0.2 ml normal or inj. rabbit serum.

TABLE II. Inhibition of Human Clotting Factors by Rabbit Plasma.

Test	Normal rabbit $BaSO_4$ plasma	Inj. rabbit plasma $BaSO_4$	N*	PTC*	ProC*
Plasma Anticoagulant	0	2+	—	—	—
Anti-Prothrombin	0	tr	0	0	0
" Proconvertin	0	3+	0	0	3+
" Proaccelerin	0	0	0	0	0
" AHF	0	tr	0	0	tr
" PTC	0	4+	0	4+	tr
" Hageman Factor	0	0	0	0	0
" Fibrinogen	0	0	—		
" Thromboplastin	0	0	—		
Brain†	0	0	—		
Plasma	slight	slight	—		

* Samples of $BaSO_4$ treated rabbit plasma incubated 18 hr with equal volumes of normal human plasma (N), PTC deficient plasma (PTC) or proconvertin deficient plasma (ProC) and centrifuged at 20,000 rpm before testing.

† Tested on whole plasma.

possible that the assays are falsely low due to inhibition of PTC and proconvertin in the human substrates. That AHF and PTC in injected rabbits are actually normal is shown by the normal thromboplastin generation (Fig. 1, part 1) and by the assays on rabbit plasma fractions, prepared to contain the factor in question and to be free of inhibitors.

Inhibitory activity of rabbit plasmas to human clotting factors. Table II shows that the normal rabbit $BaSO_4$ plasma had no inhibitory effects other than a slight inhibition on formed plasma thromboplastin. $BaSO_4$ treated plasma from the injected rabbit prolonged normal human plasma recalcification time (plasma anticoagulant) and markedly inhibited human proconvertin and PTC. It also produced a slight diminution of prothrombin and AHF activity, but had no effect on proaccelerin, Hageman factor, fibrinogen or brain thromboplastin. In an attempt to separate the anti-PTC and anti-proconvertin activities of the injected rabbit plasma, equal volumes were incubated 18 hrs. (4°C) with normal human, PTC deficient, and hypoproconvertinemic plasmas. Tests on these mixtures showed that incubation with normal plasma removed all inhibitory effects, incubation with PTC-deficient plasma removed anti-proconvertin and incubation with proconvertin deficient plasma removed most of the anti-PTC.

Fig. 1 also demonstrates the inhibitory effects of the injected rabbit plasma. The first part of the figure shows that $BaSO_4$ plasma and serum from the injected rabbit generate plasma thromboplastin as effectively as those from the normal rabbit. In the second part the normal $BaSO_4$ rabbit plasma is effective when mixed with human serum, showing that rabbit AHF (and proaccelerin?) react normally with human PTC (and proconvertin?). On the other hand, the injected rabbit $BaSO_4$ plasma-human serum mixture does *not* generate thromboplastin. This can be interpreted either as lack of AHF in the injected rabbit or as inhibition of PTC (+ proconvertin) in the human serum. The third part of Fig. 1 shows that the activity of human $BaSO_4$ plasma (AHF + proaccelerin) is not inhibited by the injected rabbit serum.

Precipitin reactions. Normal rabbit plasma did not give precipitin reactions with human plasma. Plasma and serum obtained from the rabbits 12 days after the last injection of "PTC," either whole or $BaSO_4$ treated, gave markedly positive precipitin reactions with the "PTC" fraction, normal plasma or serum and PTC deficient plasma. Plasma obtained 12 days later still had marked anti-PTC and anti-proconvertin activity, but the precipitin reaction was far weaker and only a marked cloudiness without flocculation was observed. Table III shows that when this rabbit plasma was treated with $BaSO_4$ and then adsorbed with human plasmas of various types, the pre-

TABLE III. Precipitin Reactions.

Human material	Inj. rabbit plasma 7-11-56 $BaSO_4$	7-23-56 $BaSO_4$	N*	PTC*	ProC*
Normal plasma	4+	2+	0	+	+
" serum	4+	+	—	—	—
"PTC" fraction	4+	2+	—	—	—
PTC deficient plasma	4+	2+	0	0	+
Proconvertin deficient plasma	—	+	0	+	0

* See footnote Table II.

cipitin reactions were affected in the same way as the inhibitor reactions.

Discussion. The experiments illustrate clearly that the rabbit and human clotting factors studied have similar and interchangeable biological activities, but that the PTC and proconvertin of man and of rabbits are immunologically dissimilar. Following injections of a human serum fraction containing PTC and small amounts of proconvertin, rabbits developed inhibitors to these human factors but not to their own. Equally heavy precipitin reactions were observed when this rabbit antiserum was incubated with normal or with PTC deficient plasma, indicating the presence in the rabbit antiserum of antibodies other than anti-human PTC. Antiproconvertin was also identified. Separation of the antiproconvertin and anti-PTC activities was achieved by differential adsorption.

These experiments suggest that it may be possible to prepare antisera specific to the various human clotting factors. With sufficient purification of the antigens, antibodies of high specificity and in high titer may result which could be employed in assay methods independent of the formation and titration of thrombin.

Summary. Rabbits injected with human serum "PTC" developed inhibitors to human PTC and also to human proconvertin. These two activities could be separated by differential adsorption.

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1. Lawrence, J. S., and Johnson, J. B., *Tr. Am. Climat. and Clin. Assn.*, 1941, v57, 223.

2. Munro, F. L., and Munro, M. P., *J. Clin. Invest.*, 1946, v25, 814.

3. Craddock, C. G., and Lawrence, J. S., *Blood*, 1947, v2, 505.

4. Conley, C. L., Rathbun, H. K., Morse, W. I., (II) and Robinson, J. E., Jr., *Bull. Johns Hopkins Hosp.*, 1948, v83, 288.

5. Soulier, J. P., and Burstein, M., *Blood*, 1948, v3, 1188.

6. Frommeyer, W. B., Epstein, R. D., and Taylor, F. H. L., *ibid.*, 1950, v5, 401.

7. Singer, K., Mond, E., Hyman, J., and Levy, R. C., *ibid.*, 1950, v5, 1135.

8. VanCreveld, S., Hoorweg, P. G., and Paulssen, M. M. P., *ibid.*, 1951, v6, 233.

9. ———, *Nederland. Tydschr. Geneesk.*, 1952, v96, 2745.

10. Spaet, T. H., and Kinsell, B. G., *Stanford M. Bull.*, 1954, v12, 246.

11. Hougie, C., and Fearnley, M. E., *Acta Haemat.*, 1954, v12, 1.

12. Hougie, C., *Brit. Med. Bull.*, 1955, v11, 16.

13. Lewis, J. H., and Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED. 1953, v82, 445.

14. Goldstein, R., Gelfand, M., Sanders, M., and Rosen, R., *J. Clin. Invest.*, 1956, v35, 707.

15. Lewis, J. H., Ferguson, J. H., and Arends, T., *Blood*, 1956, v11, 846.

16. Pinniger, J. L., and Prunty, F. T. G., *Brit. J. Exp. Path.*, 1946, v27, 200.

17. Hardisty, R. M., and Pinniger, J. L., *Brit. J. Haemat.*, 1956, v2, 139.

18. Richards, M. D., and Spaet, T. D., *Blood*, 1956, v11, 473.

19. Fresh, J. W., Ferguson, J. H., and Lewis, J. H., *Obst. Gyn.*, 1956, v7, 117.

20. Lewis, J. H., Ferguson, J. H., Fresh, J. W., and Zucker, M. B., *J. Lab. and Clin. Med.*, in press.

21. Ratnoff, O. D., and Colopy, J. E., *J. Clin. Invest.*, 1955, v34, 602.

22. Biggs, R., and Douglas, A. S., *J. Clin. Path.*, 1953, v6, 23.

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Effect of Halogenated Aromatic Hydrocarbons on Proteins of Rat Tissues.
(22779)

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The object of the present work is to study the effect of administration of various halogenated hydrocarbons on the protein content of rat tissues. Previous work(1-9) has indicated that the tissues are the most probable source of cysteine for the formation of mercapturic acids which are formed in the rat from various aromatic hydrocarbons. In view of the continuous exchange and renewal of tissue proteins at the expense of dietary amino acids, it appeared possible that the ingestion of aromatic hydrocarbons by the rat will interfere with the protein formation in the tissues by the interaction of the sulfhydryl groups with the administered hydrocarbons. The blocking of the sulfhydryl groups by the hydrocarbons should be reflected not only in the decrease in the protein content of the tissues but also in the decrease of the sulfhydryl and disulfide content of the proteins.

Methods. Albino rats of Sprague-Dawley strain were born and raised in the laboratory. Young rats, 30 to 38 days of age, weighed 63 to 133 g, older rats were of 86 to 186 days of age, weighing 176 to 366 g. In each case, age, sex, and weight of experimental and control rats were the same. Animals were housed in individual metabolism cages and water allowed *ad libitum*. Weights of animals and food consumption were recorded twice a week. Diet "D" had the following per cent composition: vitamin-free casein 8; sucrose 15; salt mixture 4 (U.S.P. XIV); Crisco 20; cod liver oil 5; corn starch 48. 200 mg of choline chloride and a complete mixture of all known vitamins in adequate amounts were mixed with the diet. Diet "A" contained 27% of casein and 29% of starch, the rest of the ingredients were in the same concentration as in Diet "D." The nitrogen content of both diets was determined. *Halogenated aromatic hydrocarbons.* Phenacyl bromide, p-chlorobromobenzene, benzyl chloride, and naphthalene tetrachloride were commercial products, and the purity of the products was established in the laboratory. As previously recorded by others(7), large amounts of the compounds sharply reduced food intakes, caused a rapid loss in weight, leading eventually to death of the animals. On autopsy, pulmonary hyperemia, hemorrhagic kidneys and occasionally hemorrhages in the pericardium were observed. No other abnormal histological changes in the lungs or kidneys were observed. The quantities of hydrocarbons fed to rats were reduced to amounts which could be tolerated by the animals. With the intraperitoneal injections of naphthalene tetrachloride, the dose was 100 mg (as a water suspension containing 1% "Tween 80")/100 g weight. *Analytical methods.* The nitrogen content of diets, excreta, and various organs was determined by the Conway ultramicro Kjeldahl diffusion method. In nitrogen balance studies the urine collected daily, and feces, collected separately, were analyzed twice a week. All determinations were made in at least triplicate. The results of nitrogen balance studies were charted according to Reifenstein *et al.*(10). The protein content of tissues was calculated as suggested by Addis *et al.*(11,12). In each tissue water content and nitrogen, the latter in a homogenate, were determined. Whole blood was used for the nitrogen analysis. Animals were anesthesized under ether, blood samples were taken from abdominal aorta, and organs removed and thoroughly washed of all blood. Organs of rats from the same experimental groups were pooled, and analyses made on pooled samples. The sulfhydryl and disulfide groups in blood were determined in the formic acid-hydrochloric acid digests employing the procedure of Kolb and Toennies(13). Total circulating blood volume was determined with T-1824 (Evans-Blue). The ratio of muscle to body weight had been previously determined on 12 adult male rats, with

TABLE I. Effect of Halogenated Aromatic Hydrocarbons in Growing Rat.*

No. and sex of rats	Hydrocarbon, % in diet	Intake diet	Days	Avg intake per day, g	Avg daily change, g
6 ♀	Phenacyl bromide, 0.5	D	38	5	–.85
6 ♀	p-Chlorobromobenzene, 1.0	D	52	8	.28
6 ♀	None	D	45	10	1.16
12 ♂	N. T., 0.25	D	30	6	–.70
12 ♂	None	D	30	6	.47
12 ♂	N. T., 1.0	A	30	21	3.46
12 ♂	None	A	30	21	5.60
6 ♀	N. T. in glycerin, 0.5	A	13	12	1.53
6 ♀	None	A	13	12	2.81
6 ♀	N. T. in glycerin, 0.25	D	19	7	–.84
6 ♀	None	D	19	7	1.15

* "N. T." denotes naphthalene tetrachloride. Intramusc. injections of N. T. emulsions in water or olive oil into rats fed diets D or A did not significantly affect growth rates, due, perhaps, to poor absorption of the hydrocarbon from site of inj.

a mean of 41%, the coefficient of variation $\left(\frac{\text{Standard Deviation}}{\text{Mean}}\right)$ being 10.5%. Weight of skeleton was calculated from body weight using the data of Donaldson(14).

Results. The data in Table I show that phenacyl bromide, p-chlorobromobenzene, or naphthalene tetrachloride inhibited growth of rats which ingested the 8% casein diet. Similar results, not reported here, were obtained on mice which were fed benzyl chloride, p-chlorobromobenzene, bromobenzene, or naphthalene. No significant inhibition of growth was obtained in young rats which ingested the same hydrocarbons with the 27% casein diet, although some inhibition of growth was observed in rats which were administered naphthalene tetrachloride as glycerin emulsion. The results in Table II show that the intraperitoneal injection of naphthalene tetrachloride into adult rats produced significant loss in weight, even when the animals ingested the 27% casein diet. The results on nitrogen balance in these animals are summarized in Fig. 1 and 2. The animals re-

TABLE II. Response of Adult Rats to Naphthalene Tetrachloride.

No. of rats	Naphthalene tetrachloride	Diet	Intake days	Avg daily intake	Avg daily wt change, g
6	0.25% in diet	D	58	10	–1.15
6	*Idem*	D*	10	14	4.55
5	"	D	58	12	.58
5	"	D*	10	13	2.40
6	1% in water, intraper.	A	15	10	–7.51
6	None	A	16	20	2.71
6	In glycerin, intraper.	A	17		–1.05
6	None	A	43		.62

* 0.12% of L-cystine in diet.

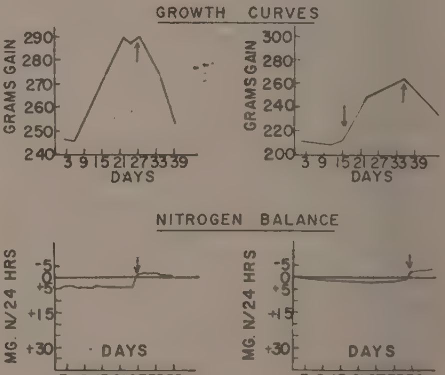

FIG. 1

FIG 2

FIG. 1 and 2. Effect of naphthalene tetrachloride on gain in wt and nitrogen balance in rats. Data represent avg results obtained on 6 male rats 128-132 days old. Upper curves are wt curves; lower curves represent nitrogen balance. Animals fed same diet for 14 days before collection of excreta. Animals represented by left 2 curves were fed Diet A plus 0.25% naphthalene tetrachloride. Arrow indicates time at which animals were continued to be fed Diet A but naphthalene tetrachloride was inj. intraper. The right 2 curves represent animals which were fed Diet D plus 0.25% naphthalene tetrachloride. First arrow indicates time at which L-cystine was added to diet. Second arrow indicates time at which naphthalene tetrachloride was inj. intraper. to rats while feeding of Diet D was continued.

TABLE III. Protein of Tissues of Rats Which Received Naphthalene Tetrachloride Intraperitoneally for 12 Days, Calculated in g per Rat per 310 cm² of Body Surface.*

	Group 1 Diet A	Group 3 Diet A + N.T.	Change in protein content, %	Group 2 Diet D	Group 4 Diet D + N.T.	Change in protein content, %
Liver	1.57	1.49	– 5	1.41	1.56	11
Kidneys	.26	.22	–14	.22	.24	9
Digestive tract	.88	.50	–42	.72	.67	– 7
Muscle	13.85	8.40	–39	12.87	10.89	–13
Lungs	.13	.11	–15	.10	.13	30
Spleen	.07	.04	–41	.05	.04	–20
Heart	.11	.08	–27	.12	.09	–25
Blood	1.99	1.22	–38	1.69	1.26	–25
Pelt	8.02	7.09	–11	6.21	4.25	–31
Skeleton	1.55	1.34	–13	1.46	1.42	– 3
Carcass	7.88	5.57	–29	7.30	5.56	–23
Total	36.31	26.06	–23	32.13	26.10	–19

* The data were obtained on pooled tissues of animals in each group. Nitrogen balance of these animals during the 12 days of N. T. inj. is indicated in Fig. 1 and 2 by the respective arrows at extreme right portion of figures.

mained in positive nitrogen balance while ingesting diet "A" or "D" with naphthalene tetrachloride. However, when naphthalene tetrachloride was injected a definite negative nitrogen balance was observed. After 12 days of injection of naphthalene tetrachloride the animals were sacrificed, the organs were analyzed for protein content, and the results are shown in Table III. It will be noted that a loss of protein has occurred in all tissues examined, except in animals which ingested diet "D." In the latter case an increase in the protein content of the liver, kidneys, and lungs was noted. The data in Table IV suggest that a decrease in the sulfhydryl and particularly in the disulfide content of the rat blood has occurred during the injection of naphthalene tetrachloride.

Discussion. Loss in weight of rats ingesting hydrocarbons together with the 8% casein diet does not appear to be due entirely to the drop in food consumption, as was indicated by paired feeding experiments. The prompt resumption of growth on incorporation of L-cystine into the hydrocarbon-containing diet suggests interference of the hydrocarbon with the utilization of cystine (cysteine). This inference is supported by the apparent decrease in the -SH and S-S content of the blood during the intraperitoneal administration of naphthalene tetrachloride. It is of interest to note that Tsuji(15) and Nakashima(16) observed a decrease in the free and "bound" cysteine of the eye lens proteins as well as of glutathione of the liver and eye lens of rabbits which were fed naphthalene. As has been established, naphthalene undergoes *in vivo* conjugation with cysteine and is in part excreted in the urine of animals as the naphthalene mercapturic acid. It is possible that naphthalene tetrachloride is similarly metabolized in part to a mercapturic acid derivative. The data further suggest that in the course of metabolic disposal of the hydrocarbons examined an interference with a normal metabolism of proteins in various tissues takes place which is minimized either by a high protein content of the diet or by supplementary cystine. Binding of various hydrocarbons to the tissue proteins has been emphasized in recent years. We have no direct evidence that the hydrocarbons which we examined in this study combine directly with the tissue pro-

TABLE IV. Sulfhydryl and Disulfide Content of Blood of Rats Which Receive Intraperitoneally Naphthalene Tetrachloride for 12 Days.*

Diet and hydrocarbon	Sulfhydryl for 310 cm² of original body surface	Disulfide for 310 cm² of original body surface
A	1.36	19.52
A + N. T.	.68	5.22
D	1.12	12.93
D + N. T.	.71	6.75

* Sulfhydryl and disulfide expressed in mg as cysteine and cystine respectively/rat.

teins, although the possibility exists that the hydrocarbons interacted with the -SH or S-S groups of the proteins in a manner which prevented the detection of these groups by the analytical procedures which we employed. The binding of the hydrocarbons to the proteins, particularly in liver, could conceivably be reflected in the alteration of their metabolic availability to other organs and in the protein content of these organs.

Summary. Phenacyl bromide, p-chlorobromobenzene, or naphthalene tetrachloride inhibit growth of rats which ingested an 8% casein diet. The inhibition was alleviated by supplementary cystine, or by a 27% casein diet. Nitrogen balance remained positive in rats which ingested the 27% casein diet or the 8% casein diet which was supplemented with cystine. Intraperitoneal injection of naphthalene tetrachloride into adult rats on the high or low protein diet induced a negative nitrogen balance, accompanied by a decrease in the -SH and S-S content of the whole blood and in the protein content of practically all tissues. The implication of these observations is discussed in terms of possible interference of the hydrocarbons with the protein metabolism of the rat via direct binding of the hydrocarbons to the proteins through the -SH or S-S groups.

1. Stekol, J. A., *J. Biol. Chem.*, 1935, v110, 463.
2. ———, *ibid.*, 1936, v113, 675.
3. ———, *ibid.*, 1937, v117, 619.
4. ———, *ibid.*, 1937, v121, 87-93.
5. ———, *ibid.*, 1939, v127, 131.
6. ———, *ibid.*, 1939, v128, 199.
7. ———, *ibid.*, 1947, v167, 637.
8. ———, *Arch. Biochem.*, 1943, v2, 151.
9. Williams, R. T., *Detoxication mechanisms*, New York, 1949, 60-67.
10. Reifenstein, E. C., Albright, F., and Wells, S. L., *J. Clin. Endocrinol.*, 1945, v5, 367.
11. Addis, T., Foo, L. J., and Lew, W., *J. Biol. Chem.*, 1936, v115, 111.
12. Addis, T., Foo, L. J., Lew, W., and Yuen, D. W., *ibid.*, 1936, v113, 497.
13. Kolb, J. J., and Toennies, G., *Anal. Chem*, 1952, v24, 1164.
14. Donaldson, H. H., *Data and reference tables, Philadelphia*, 2nd ed., 188, 1924.
15. Tsuji, T., *J. Biochem., Japan*, 1932, v15, 33.
16. Nakashima, T., *ibid.*, 1934, v19, 281.

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Coagulation-Promoting and Inhibitory Properties of Modified Thrombin Preparations.[‡] (22780)

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The fibrinogen-clotting activity of thrombin is known to be destroyed by heating above 60°C(1). As will be shown here, such heated preparations have profound effects on various phases of the coagulation mechanism.

Materials and methods. Tropical thrombin;[*] bovine fibrinogen, Fraction I;[†] human plasma; bovine plasma; human platelet material; degradation products of thrombin. Human plasma was prepared from normal blood which had been treated with a solution of sodium oxalate (1 part of 0.1 M oxalate solution in water to 9 parts of blood) and was centrifuged at 32,000 g for 30 minutes at room temperature. Bovine plasma was prepared in a similar manner. Human platelet material was prepared by the method described previously(2). Inhibitors of thrombin were prepared by heating solutions of thrombin in isotonic saline in the waterbath for varying periods at temperatures ranging from 40°C to 100°C. In most of the work reported here, the material studied was ob-

[‡] This investigation was supported by the Atomic Energy Research Contract AT(30-1) 1275 and by Grant No. C937 from the U. S. Public Health Service.

[*] Parke Davis and Co., Detroit, Mich.

[†] Armour and Co., Chicago, Ill.

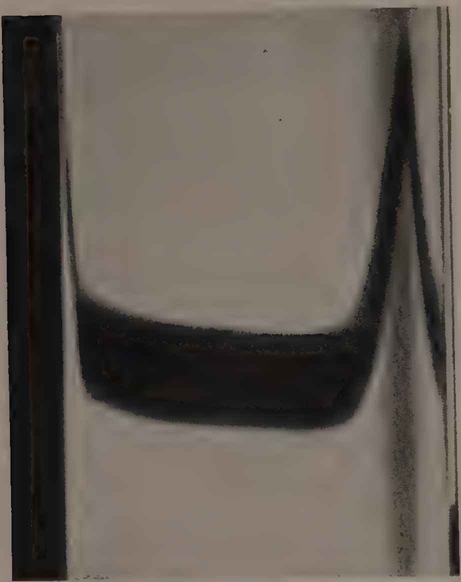

FIG. 1. Ultracentrifugal pattern of supernatant obtained from boiled thrombin preparation.

tained by heating thrombin at 100°C for 30 minutes. The thick coagulum which formed was removed by centrifugation. The supernatant (B.T.) was precipitated with 9 volumes of 95% ethyl alcohol. The white precipitate (A.T.) was collected by centrifugation, dried *in vacuo* and redissolved in water, physiological saline or imidazole buffer at a pH of 7.4. The activity obtained by treating one unit of thrombin as described above will be referred to as one unit of B.T. or A.T., respectively.

The following determinations were carried out on plasma to which B.T., A.T. or a blank (saline or buffer) had been added: recalcification time according to a modification(3) of the method of Quick(4); thromboplastin generation test(5) modified as described previously(6). The thrombin time was determined by forcefully ejecting 0.1 cc of thrombin solution into test tube (7 mm) containing 0.1 cc of buffer or solution of heated thrombin preparation and 0.2 cc of a solution of 400 mg % fibrinogen in imadazole buffer. The first appearance of a fibrin thread was taken as the end-point. Thrombin generation, estimated by the method previously described (7), was modified in the following manner. 4 cc of a 0.25 M solution of calcium chloride in water were added to 2 cc of oxalated plasma at 37°C. Aliquots of 0.1 cc of this mixture were aspirated into tuberculin syringes containing 0.1 cc of B.T., A.T. or a

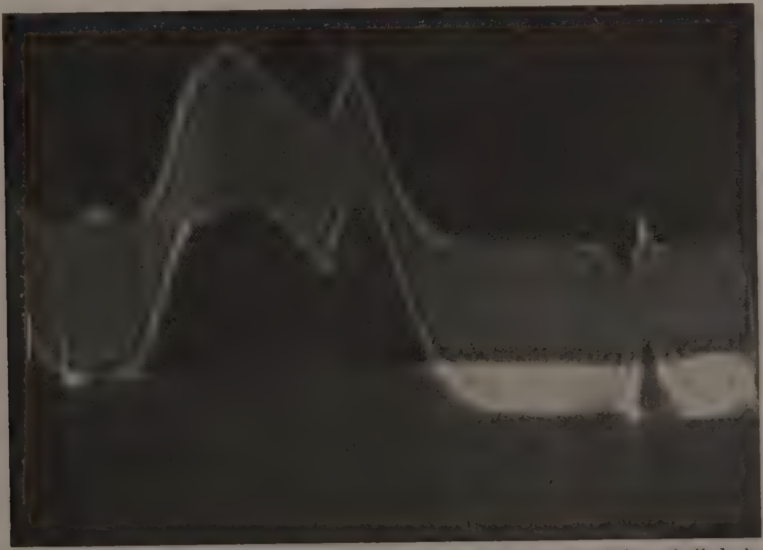

FIG. 2. Free boundary electrophoretic pattern of supernatant obtained from boiled thrombin preparation.

TABLE I. Effect of Varying Concentration of A.T. on Recalcification Time of Bovine Plasma.

A.T. units*	Clotting time, sec.
0	157
1	165
10	172
100	176
500	192
1000	265
5000	600
10,000	600

* A.T. = Alcohol precipitate. One unit of A.T. is the amount obtained from one unit of thrombin.

blank at minute intervals. The contents of the syringe were ejected forcefully into test tube (7 mm) containing 0.2 ml of a solution of 400 mg% fibrinogen in buffer. The tube was agitated by manual rotation at 37°C. The first appearance of fibrin strands was recorded as the end-point.

TABLE II. Effect of Varying Concentrations of A.T. on Recalcification Time of Human Plasma.

Duration of storage of plasma	Control (Imidazole buffer)	500 units* of A.T.†	250 units of A.T.	125 units of A.T.	62.5 units of A.T.	31 units of A.T.
	(sec.)					
Fresh plasma	220	485	310	175	210	240
Stored plasma (24 hr)	185	215	100	97	125	155
" " (1 wk)	240	305	270	186	197	236
" " (2 ")	190	115	115	90	130	150
" " (3 ")	160	118	115	110	128	—

* 1 unit of A.T. is the amount obtained from 1 unit of thrombin.
† Alcohol precipitate.

Results. Chemical characteristics: Activities in supernatant (B.T.) obtained after centrifugation of heated bovine thrombin solution, and the precipitate (A.T.) obtained by adding ethyl alcohol to B.T. were non-dialyzable, contained peptide bonds (Biuret test), carbohydrate (Orcinol test) and amino sugars (Morgan Elson reaction). Free reducing groups were not detected. In the analytical ultracentrifuge, a uniform peak is observed (Fig. 1) which differs markedly from that given by the starting material. On free boundary electrophoresis a pattern is obtained which resembles the starting material in terms of the general relation of the major peaks to each other, but the rate of migration is considerably reduced in the modified preparation (Fig. 2).

The yield of A.T. amounts to approximately 5% of the weight of the starting material.

Coagulation studies: High concentrations of heated thrombin or the alcohol precipitate inhibited the clotting of recalcified bovine plasma. Acceleration was not observed at any concentration of these materials (Table I).

Coagulation of recalcified human plasma, however, was accelerated by B.T.‡ or A.T.§ except at high concentrations, which may cause inhibition. Low concentrations were ineffective. (Table II). It appears that these effects are more marked with stored plasma.

It can be seen from Table III that the accelerating activity of B.T.‡ decreases as the lengths of time for which it is kept at 100°C increases. Addition of calcium ion leads to partial reactivation.

TABLE III. Effect of Varying the Period for Which Thrombin Is Heated at 100°C on Activity of A.T. in the Clotting of Human Plasma. (Control without addition of thrombin derivative—347 seconds.)

Time of heating thrombin at 100°C, min.	Clotting times (sec.) Without added calcium	With added calcium
0	1	1
5	85	30
10	116	57
20	600	105
30	600	132
60	600	210

‡ Supernatant obtained from heated thrombin preparation.

§ Alcohol precipitate from supernatant of heated thrombin preparation.

TABLE IV. Effects of Pre-incubating Human Plasma with 300 Units of A.T. for Varying Periods Prior to Addition of Ca^{++}.

Time of pre-incubation, min.	Clotting times, sec.
0	190
2	18
4	9
6	5
10	2

Rate of thromboplastin generation was increased by B.T.[‡] or A.T.[§] except at high concentration of these materials, which caused inhibition. (Table V).

TABLE V. Effect of Varying Concentration of A.T.* on Generation of Thromboplastin in Human Plasma.

Time of incubation of mixture, min.	Control	A.T.* 300 units†	A.T. 60 units	A.T. 10 units
	(sec.)			
1	88	58	42	50
3	43	30	12	12
4	—	—	11	11
5	13	18	11	11
6	10	17	11	11
7	10	16	11	12
8	11	15	12	—
9	—	16	—	—

* Alcohol precipitate obtained from supernatant of heated thrombin preparation.

† One unit of A.T. is the amount obtained from one unit of thrombin.

Inhibitory effects of heated thrombin preparations on coagulating activity of native thrombin. When bovine thrombin was used in "thrombin time" determinations on human and bovine plasma, its coagulating activity was inhibited by B.T.[‡] and A.T.[§]

TABLE VI. Effect of Varying Concentration of A.T.* on Activity of Bovine Thrombin Added to Human and Bovine Plasma.

A.T. units*	Human plasma, sec.	Bovine plasma, sec.
10,000	—	255
5000	—	160
1000	47	28
500	38	25
100	29	19
10	25	16
1	25	16
0	25	16

* Alcohol precipitate obtained from supernatant of heated thrombin preparation. One unit of A.T. is the amount obtained from one unit of thrombin.

In the thrombin generation test, B.T.[‡] and A.T.[§] produced inhibition in the bovine test system, but not in the human system. Representative findings are tabulated in Table VII.

TABLE VII. Effect of A.T. on Activity of Thrombin Generated in Bovine and Human Plasma.

Time of incubation, min.	Bovine plasma Control	Bovine plasma 500 units* A.T.† added	Human plasma Control	Human plasma 500 units* A.T.† added
	(sec.)			
2	205	—	212	240
3	63	—	135	173
4	30	313	43	38
5	30	240	32	30
6	24	176	30	26
7	24	99	30	25
8	24	72	25	25
9	25	65	25	22
10	29	69	30	25
12	—	75	30	27

* One unit of A.T. is the amount obtained from one unit of thrombin.

† Alcohol precipitate obtained from supernatant of heated thrombin preparation.

Discussion. The exposure of thrombin to temperatures above 60°C has been found to lead to loss of its ability to clot fibrinogen. The data presented here refer to the alcohol precipitate obtained from the supernatant of heated bovine thrombin preparations, but essentially the same results were obtained when the supernatant prior to the addition of alcohol was used. The data indicate that the redissolved alcohol precipitate (AT) obtained from heated thrombin solution has a number of effects on the various stages of the coagulation mechanism. None of these effects is due to a true thrombin activity, as evidenced by failure to clot fibrinogen. 1. The effects of the alcohol precipitate obtained from heated thrombin preparations on the recalcification time may be related to the increased rate of the generation of thromboplastin. This aspect of thrombin activity appears to differ qualitatively from its coagulant effects on fibrinogen and is more stable than the latter. 2. The effects of incubation of heated thrombin preparations with plasma prior to the addition of calcium are not understood. 3. The alcohol precipitate obtained from solutions of heated thrombin preparations inhibited bovine thrombin. This inhibitor appears to be different from the antithrombic activities described so far(8). It should be noted that the considerably lower concentrations of thrombin derivatives prepared from bovine thrombin were sufficient to inhibit the latter, than were required to inhibit thrombin. This species-specific inhibitory effect may be related to differences between bovine and human thrombin and to the ease with which the respective enzymes can be inhibited by the same inhibitor. On the other hand, the possibility may also be considered that the modification of an enzyme may result in a derivative which will inhibit preferentially the specific enzyme from which it has been prepared.

The heated thrombin preparations and the redissolved alcohol precipitates have been administered intravenously to rabbits and guinea pigs without toxic or thromboembolic sequelae.

Summary. An alcohol precipitate obtained from heated bovine thrombin preparations promoted the early phases of the coagulation mechanism, but did not clot fibrinogen in the manner of the unheated preparation. These materials inhibited bovine thrombin at lower concentrations that human thrombin in the clotting of fibrinogen.

1. Seegers, W. H., *J.B.C.*, 1940, v136, 103.

2. Klein, E., Arnold, P., Earl, R. T., and Wake, E., *N.E.J.M.*, 1956, v254, 1132.

3. Klein, E., Farber, S., Freeman, G., and Fiorentino, R., *Blood*, 1956, xi, 910.

4. Quick, A. J., *Am. J. Med. Sci.*, 1941, v210, 469.

5. Biggs, R., and Douglas, A. S., *J. Clin. Path.*, 1953, v6, 23.

6. Klein, E., and Fiorentino, R., PROC. SOC. EXP. BIOL. AND MED.,

7. Biggs, R., and Macfarlane, R. G., *Human Blood Coagulation*, Blackwell Scientific Publications.

8. Johnson, J. F., and Seegers, W. H., *The Coagulation of Blood*, Editor, L. M. Tocantins, Grune and Stratton, New York, 1955.

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Antifibrinolytic Activity of Derivatives of Fibrinolysin.‡ (22781)

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Fibrinolysin and its inhibition and activation have been intensively investigated in recent years in relation to coagulation and hemostasis. The clinical investigation of bleeding patients(1) as well as the fractionation of lyophilized human platelet material suggested the presence of an inhibitor of fibrinolysin in these materials. Antifibrinolysin, moreover, has been demonstrated previously in bovine platelets(2).

In the search for specific inhibitors of fibrinolysin, an attempt was made to produce an inactive derivative which would specifically inhibit the enzyme.

Materials and methods. Bovine fibrinolysin* was suspended in water, saline or buffer (imidazole pH 7.2), and heated at 90°C for periods varying from 5 to 30 min. Coagula formed during the heating were removed by centrifugation at 3,000 g for 10 min. The supernatant (M.F.) was precipitated with 3 volumes ethyl-alcohol to yield a white, water-soluble material (MFP). The amount of MFP obtained was approximately 4.5% of the weight of the starting material. The fibrin-plate method previously described (3) was used for the determination of fibrinolytic activity. The method was modified, however, in regard to times and temperatures of incubation. Human fibrinolysin activity was obtained by activating normal human serum with Streptokinase (Varidase).*

Results. The soluble material remaining after heating bovine fibrinolysin at 90°C was

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* We acknowledge with gratitude the generous sample received from Dr. Eugene C. Loomis, Parke Davis and Co., Detroit, Mich.

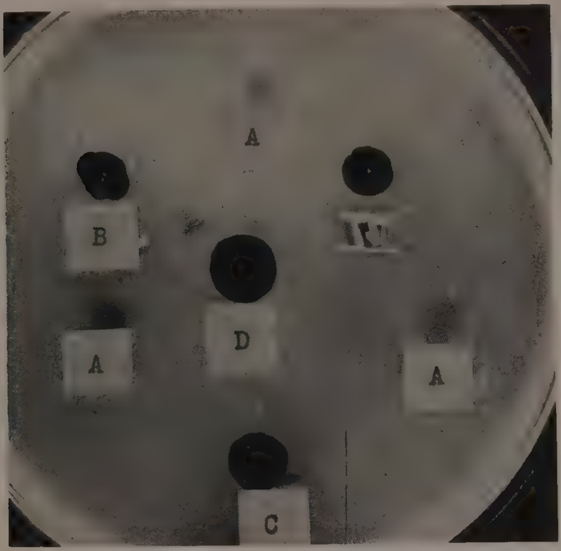

FIG. 1. A. 10 mg/cc active fibrinolysin solution mixed 1:1 with MF derived from 10 mg/cc of fibrinolysin. B. 10 mg/cc active fibrinolysin diluted 1:1 with saline. C. 10 mg/cc active fibrinolysin (undiluted). D. 20 mg/cc active fibrinolysin (undiluted).

insoluble in alcohol and gave a strongly positive orcinol reaction, indicating the presence of carbohydrate.

Both the supernatant (M.F.) as well as the material precipitated from it by alcohol (MFP), inhibited the activity of fresh fibrinolysin, as illustrated in Fig. 1.

In another experiment with MFP[†] the fibrin plates were incubated for 48 hours. Lysis of fibrin started at the point of application of fibrinolysin and spread to other areas of the plate. The area on which MFP had been applied (Fig. 2 and Fig. 3) was protected from lysis, while despite some lysis at the center of the site of application of a mixture of active and modified fibrinolysin, the surrounding fibrin was not lysed. Qualitatively similar results were also obtained with the supernatant (MF) obtained from heated fibrinolysin.

The effect of heating for various periods of time was investigated. Optimal inhibition was obtained with materials heated for 5 to 10 minutes, while heating for more than 20 minutes tended to decrease the inhibitory activity.

When modified bovine fibrinolysin was tested against human fibrinolysin (*i.e.*, serum activated with Streptokinase), fibrinolytic activity was not antagonized (Table I).

Discussion. Products obtained by heating bovine fibrinolysin preparations were found to inhibit the activity of the unmodified enzyme. The same material did not appear to affect the fibrinolytic activity of human serum treated with streptokinase. It should be noted that the successful formation of inhibitors by heat denaturation varied with the nature of the starting material and the procedures employed, in a manner not understood at the present time.

Analogous inhibitory phenomena have been

TABLE I. Effect of MFP on Human Serum Fibrinolysin.

	Avg diameter of lysed area (mm)
Activated human serum 1:1 with 20 mg MFP[*]/cc	16.5
Idem, with 10 mg MFP/cc	16.0
" with saline	16.2

[*] MFP refers to the alcohol precipitate, *i.e.*, 20 mg of dried precipitate/cc of physiological saline.

[†] Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

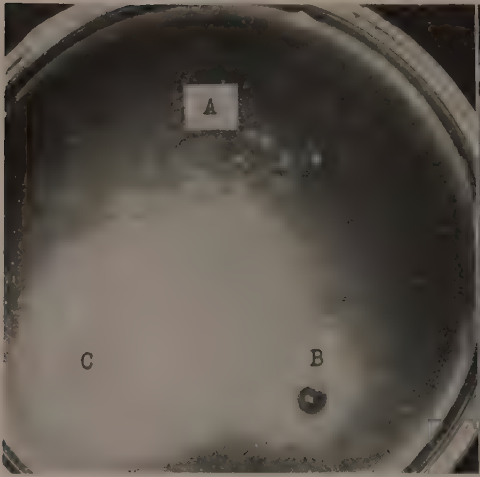

Fig. 2

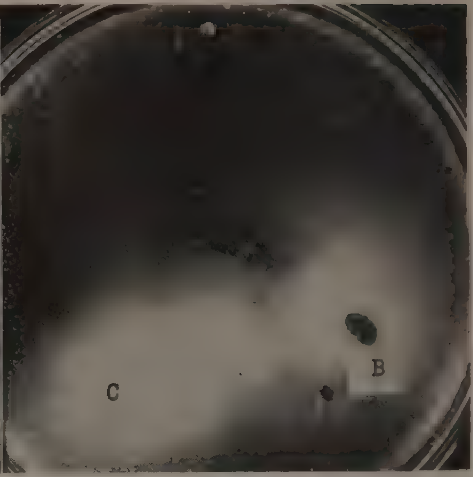

Fig. 3

FIG. 2. A. Fibrinolysin, 20 mg/cc, diluted 1:1 with saline. B. Fibrinolysin, 20 mg/cc, diluted 1:1 with MFP,* 20 mg/cc. C. MFP, 20 mg/cc.

FIG. 3. A. Fibrinolysin, 20 mg/cc, diluted 1:1 with saline. B. Fibrinolysin, 20 mg/cc, diluted 1.1 with MF† obtained from 20 mg fibrinolysin/cc. C. MF, 20 mg/cc.

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* 20 mg of dried alcohol precipitate were dissolved in 1 cc of physiological saline.

† MF refers to supernatant obtained from heating a solution of 20 mg of fibrinolysin/cc.

observed in parallel studies of thrombin and in preliminary studies with a number of other enzymes which are not related to the coagulation mechanism.

The failure of an inhibitor derived from a bovine fibrinolysin preparation to affect human fibrinolysin activity (Table I) is of interest. Studies are in progress to determine the species-specificity of this inhibitory effect, particularly in view of the known differences between functionally similar enzymes derived from different tissues and different species.

Summary. Derivatives of bovine fibrinolysin have been prepared which inhibited the enzyme from which they are derived, but did not inhibit human fibrinolytic activity.

1. Klein, E., Farber, S., Djerassi, I., Toch, R., Freeman, G., and Arnold, P., *Pediatrics*, 1956, v49, 517.

2. Johnson, S. A., and Schneider, C. L., *Science*, 1953, v117, 229.

3. Holburn, R. R., *The Coagulation of Blood, Methods of Study*, Editor, L. Tocantins, Grune and Stratton, N. Y., 1955, p161.

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Effect on Experimental Avian Atherosclerosis of Dietary Oils and Gallogen. (22782)

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(Introduced by Camillo Artom)

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The diethanolamine salt of the mono-(+)-camphoric acid ester of α, 4-dimethylbenzyl alcohol (Gallogen) has been reported(1) to impede serum and tissue cholesterol elevations in the cholesterol-fed cockerel. Recently, clinical(2-7) and experimental(8-12) aspects of the relationship between the degree of unsaturation of dietary fats and their effects on serum cholesterol have begun to be explored. The experiment reported here was designed to show what effect the feeding of fats of various degrees of unsaturation, singly and with Gallogen, would have on atherogenesis in the cholesterol-fed cockerel.

Method. The experimental arrangements and procedure were as previously described (1). Twenty-one-day-old cockerels were placed on a diet of Purina Growing Mash and 1% Cholesterol and divided into 6 groups, which received additional materials as follows: Group I, 8% Peanut Oil;* Group II, 8% Coconut Oil;† Group III, 8% Sardine Oil;‡ Group IV, 8% Peanut Oil, 0.1% Gallogen; Group V, 8% Coconut Oil, 0.1% Gallogen; Group VI, Sardine Oil, 0.1% Gallogen. An edible antioxidant, butylated hydroxyanisole (Eastman), 0.01%, was added to the sardine oil and, as a control, to the coconut oil. The diets were mixed every 2 weeks, except for the sardine oil, which was refrigerated and mixed weekly.

Results. Food consumption was equal for all the groups. During the experimental period (Feb.-June) there were 2 deaths. One occurred in Group IV during the seventh week and was of unknown cause; the other was in Group I during the eleventh week and was due to anemia complicated by endocarditis.

TABLE I. Mean Serum Cholesterol Levels in mg per 100 ml.

Wk	Group* I	II	III	IV	V	VI
0	151	151	155	161	149	147
1	497	660	532	511	618	317
2	879	1025	769	725	889	524
3	939	850	910	798	1014	598
4	1113	1002	996	891	784	556
5	1039	934	974	877	814	534
7	1177	1057	886	1058	834	489
9	1081	1061	864	927	919	476
11	1252	1106	469	938	1143	245
12	1475	1154	633	1076	1259	366
13	1331	1001	838	1029	1264	485
14	1304	1118	1123	1014	1046	447
15	1617	1273	1219	1097	1092	568
16	1642	1339	1372	1143	1054	623

* Groups I and II had 8 birds, the remaining groups had 9 each.

The averages of weekly (or fortnightly) individual serum total cholesterol determinations are given in Table I. Unfortunately, 5 days before the blood samples were obtained at the end of week 11, the supply of sardine oil was exhausted. The two groups on sardine oil were fed regular mash until their experimental diet could be resumed at the end of week 11. It can be seen that the serum cholesterol levels of both groups receiving sardine oil declined sharply during this time, but rose again when the experimental ration was resumed. This interruption, although relatively brief, causes the succeeding serum cholesterol figures for Groups III and VI to be somewhat equivocal. The Gallogen-sardine oil group did not rise much above the level attained by week 9, but the sardine oil-alone group continued to rise sharply until the experiment was ended.

Although there were no striking differences among the average serum levels of Groups I, II, and III, the group receiving sardine oil was almost consistently the lowest of the 3, by a small margin.

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* Obtained from Welch, Holme, and Clark Co., New York City, Iodine no. 95-100.

† Obtained from Welch, Holme, & Clark Co., New York City, Iodine no. 9-11 m.p. 24°C.

‡ Obtained from Pacific Vegetable Oil Corp., Gardner color 11, Iodine no. 190-200.

TABLE II. Post-Treatment Aortic and Hepatic Cholesterol Concentrations, and Body Weights.

Group	Aortic cholesterol	Hepatic cholesterol	Avg body wt (g)
	(mg/g dry wt)*		
I	19.7 ± 4.8†	81.9 ± 8.4	1833
II	20.0 ± 7.6	74.1 ± 17.8	1561
III	20.9 ± 5.8	76.5 ± 17.0	1703
IV	14.6 ± 3.6	78.2 ± 16.5	1727
V	23.9 ± 8.3	74.7 ± 25.9	1637
VI	10.7 ± 3.6	49.7 ± 12.1	1686
Normal (12 wk, from ref. 1)	7.6 ± 1.2	20.5 ± 3.0	—

* The dry wt/wet wt ratio for aorta and liver is 0.25 and 0.42, respectively.

† ± Stand. dev. $\sigma = \sqrt{\frac{\Sigma d^2}{N}}$.

The addition of Gallogen to the rations containing the various oils resulted in a decrease in the serum cholesterol in each case. This decrease was small and inconsistent in the case of the coconut oil (saturated), larger in the case of peanut oil (moderately unsaturated), and quite distinct for sardine oil (highly unsaturated), with the reservations mentioned above.

The body weights at necropsy and the results of the tissue analyses are detailed in Table II. It is apparent that the aortic cholesterol levels were essentially the same for the 3 fat-alone groups, but were again inversely proportional to the unsaturation of the fats when these were co-fed with Gallogen. Hepatic cholesterol was affected markedly only in the group fed both sardine oil and Gallogen. Body weight was highest in Groups I and IV, receiving peanut oil, and lowest in Groups II and V, receiving coconut oil.

It is noteworthy that a separately caged group, not described in this paper but receiving a control atherogenic diet plus a drug having no apparent effect, paralleled closely the serum values of Group I (and their weekly variations) throughout the experimental period, and had aortic and hepatic cholesterol values averaging 19.1 and 84.4 mg/g, respectively. This serves to illustrate the degree of precision attained in this experiment.

Summary. 1. Dietary fats of varying degrees of saturation were no different from each other with respect to their effect on aortic or hepatic cholesterol levels of cholesterol-fed cockerels and only slightly with respect to serum levels. 2. When 0.1% Gallogen was added to the diet, serum and aortic levels were in proportion to the degree of saturation of the fat fed.

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1. King, Jr., J. S., Clarkson, T. B., and Warnock, N. H., *Circulation Research*, 1956, v4, 162.

2. Lever, W. F., and Waddell, W. R., *J. Invest. Dermat.*, 1955, v25, 233.

3. Ahrens, Jr., E. H., Blankenhorn, D. H., and Tsaltas, T. T., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 872.

4. Bronte-Stewart, B., Antonis, A., Eales, L., and Brock, J. F., *Lancet*, 1956, v1, 521.

5. Beveridge, J. M. R., Connell, W. F., Mayer, G., Firstbrook, J. B., and DeWolfe, M., *Circulation*, 1954, v10, 593.

6. Peifer, J. J., and Holman, R. T., *Arch. Biochem. Biophy.*, 1955, v57, 520.

7. Malmros, H., and Wigand, G., *Minn. Med.*, 1955, v38, 864.

8. Kummerow, F. A., *Food and Nutrit. News*, 1955, v27, 1.

9. Shapiro, S. L., and Freedman, L., *Am. J. Physiol.*, 1955, v181, 441.

10. Swell, L., Flick, D. F., Field, Jr., H., and Treadwell, C. R., *ibid.*, 1955, v180, 124.

11. Kritchevsky, D., Moyer, A. W., Tesar, W. C., Logan, J. B., Brown, R. A., Davies, M. C., and Cox, H. R., *ibid.*, 1954, v178, 30.

12. Alfin-Slater, R. B., Aftergood, L., Wells, A. F., and Deuel, Jr., H. J., *Arch. Biochem. Biophy.*, 1954, v52, 180.

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Tryptophan Content of Casein as Determined by a Rat Growth Method.[*] (22783)

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Extreme variations in values for the tryptophan content of casein have appeared in the literature(1). These have ranged from 1.68%, obtained by applying a colorimetric method to the intact protein(2) to 0.92% listed in(3). Using the colorimetric method referred to above, analysis of alkaline hydrolysates gave a value of 1.24%(4).[†] The latter value corresponds closely to those found by microbiological assay of alkaline hydrolysates, 1.20%(5), 1.24%(6), 1.18-1.24%(7), or enzyme hydrolysates 1.28%(6), 1.31%(8), and 1.20%(1). Lower values can be attributed to the extreme ease with which tryptophan is destroyed by hydrolysis procedures or to incomplete hydrolysis in the case of enzymatic methods. The higher values may result from lack of specificity of the chemical methods which have been used. In this connection, it is interesting to note that a bioassay with *Tetrahymena geleii*(9), in which preliminary hydrolysis is not required, has given results which agree closely with those of most microbiological assays.

In view of this extreme variation, probably due largely to difficulties in hydrolysis, it seemed desirable to apply an animal assay to this problem. Such assays have been widely used for most essential nutrients and it seemed particularly applicable to tryptophan, since the report by Oesterling and Rose(10) indicated that the rat's weight gain is related linearly to the L-tryptophan dosage, when the latter is varied from 0.10 to 0.15% of the diet. The chief source of error in this assay method might result from incomplete hydrolysis of the protein with accompanying incomplete absorption of tryptophan from the small intestine. This would tend to give low values but the same errors would be encountered whenever casein is employed in rat feeding experiments. On the other hand, high values might result if the free tryptophan used as the standard were not utilized as well as that released from the digested casein.

Methods. Female weanling rats weighing 55 to 65 g were obtained from the local stock colony. The experimental period was 4 weeks during which time the animals were housed in individual cages in an air-conditioned room and given food and water *ad libitum*. Weekly weight gains and daily food consumption were recorded. The basal diet contained 15% acid-hydrolyzed casein. This preparation, previously described(11), had the following amino acid composition: phenylalanine, 4.0%; histidine, 1.1%; methionine, 2.7%; arginine, 2.6%; valine, 6.2%; leucine, 8.0%; threonine, 2.9%; isoleucine, 4.7%; lysine, 5.9%; total nitrogen, 13.28%. The remaining components of the basal diet were: 4% Salts IV(12), 2% vitamin mixture, 0.1% L-histidine, 0.2% L-cystine, 5% corn oil and sucrose to make 100%. The vitamin mixture provided the following quantities per kg of diet: thiamine • HCl 2 mg, riboflavin 3 mg, pyridoxine • HCl 2.5 mg, calcium pantothenate 20 mg, choline chloride 1 g, inositol 100 mg, biotin 0.1 mg, folic acid 0.2 mg, vit. B_{12} 17 μg, and niacin 25 mg. Levels of L-tryptophan from 0.04 to 0.15% or of casein from 4 to 9% were incorporated into the basal diet with suitable reductions in the acid-hydrolyzed casein to maintain the nitrogen level of the diet at 1.99%. The L-tryptophan used was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. The casein employed in these experiments was vitamin-free Labcocasein (Borden Co., N. Y.) and con-

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[*] This study was supported by grant from U.S.P.H.S., R.G., A-801.

[†] The recent use of the higher values in the design and interpretation of nutritional experiments prompted the studies reported here. See Ebisuzaki, K., Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, 1952, v198, 63, and Hopper, J. H., and Johnson, B. C., *J. Nutrition*, 1953, v56, 303.

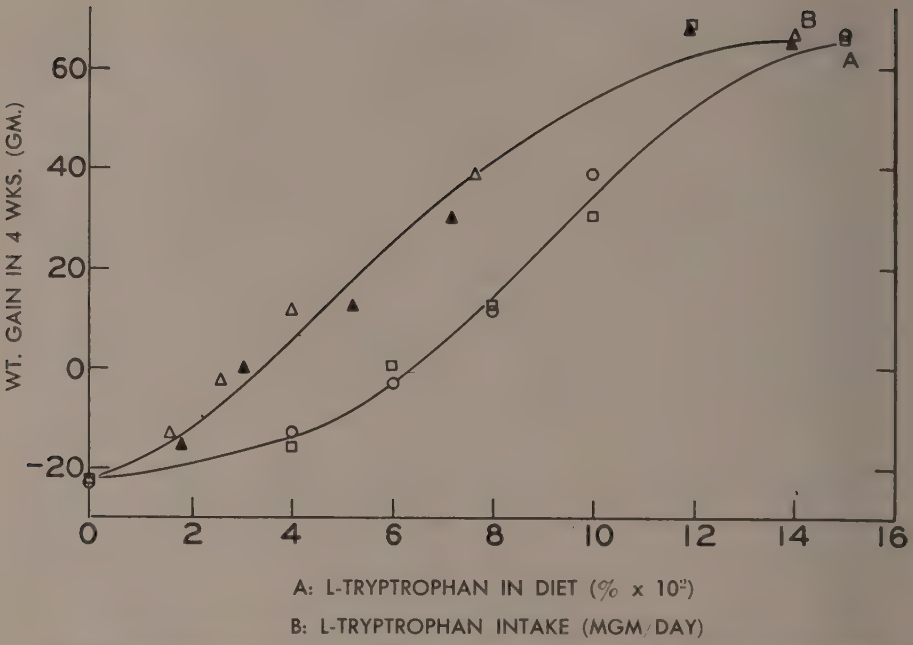

FIG. 1. Standard dose-response curves for tryptophan expressed as % of diet and as intake in mg/day.

tained 14.64% nitrogen on a moisture-free basis.

Results. Fig. 1 presents standard curves representing the data from two experiments. From the daily food consumption and the level of tryptophan in each diet, it was possible to calculate the daily L-tryptophan intake which is plotted against the growth rate in curve B. Curve A was constructed by plotting the growth rate versus the level of L-tryptophan in the diet. The experimental points from both studies are presented, but since the results were in good agreement, single standard curves were drawn. In each experiment the increase in body weight during 4 weeks for each level of casein was measured; these growth rates in conjunction with curve A provide the basis for the calculation of the per cent tryptophan in these diets and, from this, the per cent tryptophan in the casein (Column A, Table I). The most nearly linear portion of the dosage-response curve (A), which gives the most reliable value for the tryptophan present in casein, lies between 0.05 and 0.12% L-tryptophan. Food consumption data on the same animals were

TABLE I. L-Tryptophan Content of Casein.*

Exp.	Casein in diet, %	Avg wt gain 4 wk, g	Tryptophan in casein (%) A†	B†
I	4	− .5	1.56‡	1.82‡
	6	7.5	1.22	1.32
	8	36.5	1.28	1.33
		Avg	1.35 ± .08§	1.49 ± .29
II	5	−4.0	1.16	1.33
	6	8.5	1.23	1.28
	7	[illegible]	[illegible]	[illegible]
	8	38.0	1.29	1.20
	9	58.5	1.44	1.45
		Avg	1.24 ± .06	1.27 ± .14

* Two animals received each level of casein in both experiments.

† Values in column A are based on Curve A; those in column B are based on Curve B (Fig. 1).

‡ Growth data yielding these values involves consideration of the less reliable lower portion of Curves A and B.

§ Stand. error of the mean.

Calculation of these values involves the less reliable upper portions of Curves A and B.

employed to calculate the tryptophan content of casein from curve B.

The highest values (Table I) calculated from the growth rates with 4 and 9% casein diets appear to result from the use of less reliable portions of the dose-response curves, near or beyond the inflection points. In contrast to these seemingly unreliable values, extremely good agreement between the two experiments was obtained on the 6% and 8% casein diets. All of these values were in the range from 1.22 to 1.33% whether calculated on the basis of percentage of the diet (Column A) or actual tryptophan ingested (Column B). From the most reliable data of Experiment I and all of the results in Exp. II, the best value for the tryptophan content of casein appears to be 1.25%.

Further support for this value is given by amino acid imbalance studies. Experiments in this area have shown that rats develop a niacin deficiency on either a 9% casein diet (13) or purified amino acid diets containing 0.10% to 0.11% of tryptophan and levels of other essential amino acids approaching those required for optimum growth(14). If threonine or another essential amino acid is reduced to a level which is more limiting than tryptophan, the growth rate is improved. It has been found that the tryptophan content of the diet is critical in obtaining such unusual imbalance effects(11). The narrow range, 0.10 to 0.11% of tryptophan or 9% casein in the diet, over which these effects are observed may be used as a basis for roughly calculating the tryptophan content of casein. Thus a value of 1.11 to 1.22% is indicated from the data reported.

For the purposes of comparison with other values in the literature the one arrived at here corresponds to 1.37% when converted to the hypothetical 16% nitrogen basis or to 8.52 mg of tryptophan per 100 mg of casein nitrogen.

Summary. The tryptophan content of casein has been estimated using a rat growth assay method. Whether the results were calculated on the basis of the percentage of tryptophan in the diet or by using food consumption data to calculate the daily intake of tryptophan, essentially the same results were obtained. These animal assays indicate that casein (14.64% N) contains 1.25% tryptophan which is in good agreement with many reports based on microbioassays of alkaline and enzyme hydrolysates of this protein, but much lower than many commonly accepted figures obtained with chemical methods.

The technical assistance of Bonnie J. McLaughlin is gratefully acknowledged.

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1. Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, 1945, v157, 153.

2. Spies, J. R., and Chambers, D. C., *Anal. Chem.*, 1949, v21, 1249.

3. Albritton, E. C., *Standard values in nutrition and metabolism, Handbook of biological data,* 1953, p131, Wright Patterson Air Force Base, O.

4. Steers, E., and Sevag, M. G., *Anal. Chem.*, 1949, v21, 641.

5. Krehl, W. A., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, 1946, v164, 551.

6. Henderson, L. M., and Snell, E. E., *ibid.*, 1948, v172, 15.

7. Greene, R. D., and Black, A., *ibid.*, 1944, 155, 1.

8. Greenhut, I. T., Schweigert, B. S., and Elvehjem, C. A., *ibid.*, 1946, v165, 325.

9. Rockland, L. B., and Dunn, M. S., *Arch. Biochem.*, 1946, v11, 541.

10. Oesterling, M. J., and Rose, W. C., *J. Biol. Chem.*, 1952, v196, 33.

11. Henderson, L. M., Koeppe, O. J., and Zimmerman, H. H., *ibid.*, 1953, v201, 697.

12. Phillips, P. H., and Hart, E. B., *ibid.*, 1935, v109, 657.

13. Hankes, L. V., Henderson, L. M., and Elvehjem, C. A., *ibid.*, 1949, v180, 1027.

14. Koeppe, O. J., and Henderson, L. M., *J. Nutrition*, 1955, v55, 23.

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Alteration of Plasma Electrophoretic Patterns and Anaphylactic Reactions in Protein Depleted Dogs.* (22784)

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In 1939 one of us (P.F.H.) reported a means of demonstrating the limitation of hemoglobin production through protein depletion(1). Briefly this method consisted in the forcing of hemoglobin production by maintaining the dog at an anemic level by repeated bleeding, accompanying this by the intravenous administration of colloidal iron in quantities more than equal to the amount of iron removed as hemoglobin, and at the same time maintaining the animal on a diet highly restricted in its protein content. The dog has a propensity to manufacture hemoglobin at the expense of most other body proteins and, as a result, a rapid depletion of protein reserves was achieved. Preliminary studies at the time suggested that the ability of the animal to form antibodies was probably impaired as judged by the fact that the administration of a shocking dose of horse serum 2 weeks after injection of sensitizing doses did not result in a typical anaphylactic reaction. Resumption of the protein depletion work has recently been undertaken with the specific intent to prepare animals which may be suitable for homo-grafts of various tissues. The dog is usually regarded as an unsatisfactory animal for studies of the anaphylactic response, at least insofar as it can be compared with the guinea pig. Nevertheless, we have continued to use this animal for these studies for several reasons, namely: 1. the dog is more amenable to changes in diet than most experimental animals. 2. phlebotomy is considerably facilitated in the dog as compared to small animals and, 3. results of this type of study are much more easily translatable to human studies than in the cases of some of the more widely used small experimental animals. Although skin-graft type of experimentation could probably be equally as well carried out with any of the animals, such studies as survival of homologous kidney transplants are much more practicable in the dog.

We wish to present some preliminary studies relative to depletion of the plasma protein constituents of animals under such a regime. Electrophoretic patterns have been determined on these animals and are now being used as criteria to indicate whether or not the animal has been sufficiently depleted of the protein fractions to warrant an anaphylactic test. The absence or modification of the anaphylactic reaction in such depleted animals is in turn used as an indicator of the animal's readiness for the above-mentioned surgical procedures. Such studies show that, although slight to moderate reduction of the total plasma proteins occurred, marked alteration in the plasma protein makeup was clearly demonstrated.

Methods. Eight litter-mate hounds were used for depletion studies in this series. The animals were adults (over 1 year of age) and weighed from 11.7-13.6 kg initially. They were dewormed, vaccinated against distemper, and maintained on a diet of commercial dog food for 4 months prior to use to assure their status of health and to establish a relatively constant weight, blood picture, etc. During the experimental period they were fed a diet consisting of 300 g of bananas, 100 g of canned salmon, 120 g of Karo syrup, 40 ml of cod liver oil and 1 g of McCollum-Simmonds salt mixture daily. In our early studies the animals maintained or gained weight during the experimental procedure, whereas the animals cited in Table I lost weight throughout the experiment and became quite emaciated. The only obvious difference in the two experimental periods resided in the lowering of the intake of protein during the last 6 weeks of

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* Carried out under contract At-(40-1)-269 with Division of Biology and Medicine, USAEC and supported in part by grant from Josiah Macy, Jr. Foundation.

TABLE I. Anaphylaxis in Control Animals.

	Normal	Exp.
Hypernea	8/11	2/8
Salivation	8/11	4/8
Extension	5/11	6/8
Retching	8/11	4/8
Urination	11/11	3/8
Defecation	10/11	3/8
Vomiting	8/11	4/8
Collapse	5/11	1/8
Bleeding tendency	7/11	1/8
Hematuria (gross)	4/11	0/8
Other signs	0/11	1/8
Death	4/11	1/8

the experimental period in our present group, the amount of salmon muscle received in the daily diet having been reduced from 100 to 10 g. It is probable that such an alteration in protein intake may have been quite critical at this stage. Anemia was initially induced by bleeding an estimated ¼ of the blood volume (assuming 72 ml blood per kg) on each of 3 successive days. The animals were then rested for 1 day and the same amount of blood was withdrawn on the 5th day. Subsequent bleeding was such as to maintain an hematocrit value of 20-30%. Iron was administered in the form of the saccharated oxide, "Feojectin"† in quantities which were determined by previous phlebotomy to ensure that an excess existed in stores at all times. No attempt was made to quantitate the amount of hemoglobin removed but rather an estimation was made on a basis of total blood withdrawn and the hematocrit value. The depletion period lasted for 6 months. Electrophoretic studies were carried out on a portable apparatus made by the American Instrument Co., Silver Springs, Md. The plasmas were diluted with an equal volume of diethylbarbiturate buffer of pH 8.6 and an ionic strength of 0.1. The diluted plasmas were dialyzed against the buffer solution for two hours at 1-1.5°C after which the cell was filled. The duration of all runs was 90-120 minutes. Sensitization to the horse serum was accomplished by intravenous administration of 5 ml of horse serum and an additional 5 ml subcutaneously. At the end of 3 weeks 20 ml of horse serum were introduced by vein for anaphylactic test. Following administration of the shocking dose the dog was immediately untied and placed on the floor for observation. Record was kept of subsequent symptoms such as stiffening of legs, excessive salivation, bleeding tendencies, gagging, retching, vomiting, scratching, defecation and collapse. A group of 11 stock animals in apparent healthy condition was sensitized to horse serum at the same time as the test animals, using the same material. When shocking doses of horse serum were administered to this group for comparison with the experimental animals, one dog showed a minimal response inasmuch as there were no symptoms except those of excessive itching as shown by the animal scratching himself vigorously for a prolonged period. The other controls exhibited the usual symptoms such as extension of limbs, retching, collapse, etc. (Table I). Two died within 12 hours. One of the depleted dogs was extremely weak and emaciated at time of the test shock, being barely able to stand alone. Administration of the shocking dose was followed by death without

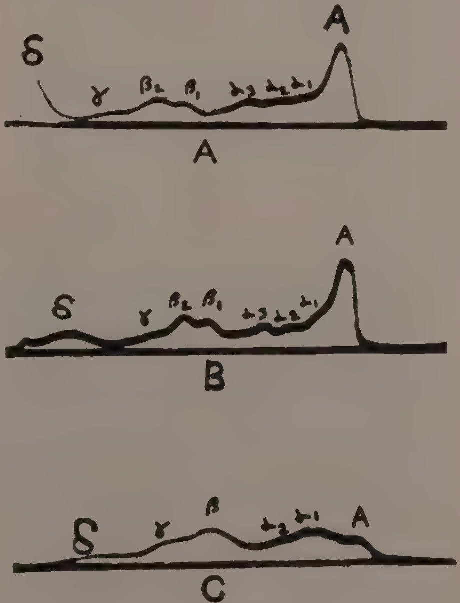

FIG. 1.

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† Supplied in generous amounts through the courtesy of Smith, Kline & French Co.

TABLE II. Total Plasma Proteins and Electrophoretic Fractions of Plasma (± S.E.), in 7 Dogs per Series.

Condition of dog	Total proteins*	A/G	Electrophoretic fractions (%)						
			Alb.	Alpha-1	Alpha-2	Alpha-3	Beta-1	Beta-2	Gamma
Normal	6.34	.85	45.9	10.3	7.7	6.9	10.4	13.3	4.8
	± .24	± .05	± 1.41	± 1.07	± 1.16	± 1.06	± .45	± .39	± .30
"	5.17	.81	44.0	10.7	9.7	6.9	11.7	16.3	5.0
	± .14	± .09	± 2.60	± 1.00	± 1.37	± 1.85	± .52	± .46	± .18
Depleted†	4.86	.12	10.5	29.7	12.5	—	37.7 ± 1.21		9.4
	± .15	± .01	± .65	± .37	± 1.19				± .98

* Determined by Semi-Micro-Kjeldahl method.

† Two dogs died during depletion regime.

the common signs of anaphylaxis within a few minutes. In the others the usual manifestations were less severe or absent and they all survived. This phase of the study will be described at length in another communication.

Results. Plasma electrophoretic patterns of the depleted animals are represented by that of dog 54-97 in Fig. 1 which is quite typical of the group.

Table II shows the plasma electrophoretic studies on these 7 litter mate hounds. In each case patterns were run at time of acquisition of the dog and again 5 months later, during which interim the animals were maintained on Purina dog chow and observed closely for abnormalities and for the establishment of baseline hematologic studies. Immediately after the second series of electrophoretic studies the litter was placed on the depletion regime for 6 months. At the end of this time another electrophoretic pattern was determined on each animal. It can be noted that there were only moderate alterations in the *total plasma proteins* with an average reduction of about 20%. This relatively small decrease in total protein content was in contrast to the marked change in the A/G ratio which was decreased from an average of 0.83 under normal conditions to 0.12 in the depleted state or a lowering of approximately 85%. The percentage of albumin ranged from 40-50% in the normal state, while in the depleted state it was reduced to values ranging from 9-13%. In each instance there was a marked increase in total α-globulin. In no case were there 3 alpha globulin peaks found in the dogs following depletion as were found in the normal state. The combined beta-1 and beta-2 globulins were increased consistently in each plasma sample following depletion, the increase amounting to about 1.5 times the value found in the normal state. The gamma globulin values were approximately doubled in the depleted state. This fraction is generally believed to contain most of the antibodies, partial depletion of which by this technic we have reason to feel is accomplished. It is known that the gamma globulin fraction as determined by electrophoresis consists of several components and so obviously a marked decrease in one element with which we are critically concerned may be entirely masked by a concomitant increase in several of the other contributing components. On the other hand it is possible that the antibody with which we are concerned exists in the β_2-globulin fraction.

Summary. 1) Dogs were depleted of body proteins by forced hemoglobin production superimposed on a low protein diet. Electrophoretic patterns indicated markedly decreased albumin values and A/G ratios in spite of relatively little change in total plasma proteins. In all instances there were marked increases in total α, β and γ globulins. 2) Anaphylactic reactions following immunization to horse serum were diminished or absent in the depleted animals as contrasted to controls.

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1. Hahn, P. F., and Whipple, G. H., *J. Exp. Med.*, 1939, v69, 315.

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Negative Ventricular Diastolic Pressure in Beating Heart Studied *in vitro* and *in vivo*.* (22785)

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Since the classic studies of Harvey(1) it has been generally accepted that diastolic filling of the heart resulted from venous return and auricular contraction. While the senior author(2) was studying the effects of anoxia and work on the glycogen content of the rat heart, it was necessary to make observations on the excised heart beating in saline. With each systole it was observed that fluid ejection from the aorta resulted in the jet propulsion of the heart in the solution and it was obvious that the heart must draw in fluid in each diastole to make possible subsequent systolic ejection; thus it was concluded that part of systolic energy was dissipated in diastole(2).

This paper presents the results of a study of pressure changes in intact and excised heart during systole and diastole.

Materials and methods. White rats weighing 300 to 450 g were anesthetized with pentobarbital 6 mg/100 g. The thorax was rapidly opened and the intact heart removed. A 20 gauge needle was inserted into the left ventricle and the needle connected to a three-way stop cock on a Statham strain gauge so that opening the stop cock recorded hydrostatic zero. The heart was placed in a pottery pie plate filled with saline. The three limb leads of the EKG were placed equidistant around the perimeter of the plate with the electrodes immersed in solution. Pressures and EKG were recorded with an Oscillograph. When desired the auricles were opened widely or excised with a pair of scissors. *In vivo* studies were performed on dogs anesthetized with pentobarbital. Artificial respiration was administered through an intratracheal tube and the chest was widely opened. Umbilical tape was placed around the superior vena cava and azygos veins. In other instances tape was placed around the pulmonary veins. The venous return to right or left ventricle could thus be diminished by tightening the ligatures. A 19 gauge needle with multiple openings in the end was located in the left or right ventricle. The atmospheric zero pressure at the needle tip could be measured by opening stop cock to the air at needle level. The distance between the needle and the highest level of the ventricle was measured to determine the hydrostatic pressure of the column of blood above the needle. The high and low pressures were then charted (Figs. 2 and 3). Electrocardiograms and pressures were recorded on the same record-

EXCISED RAT HEART

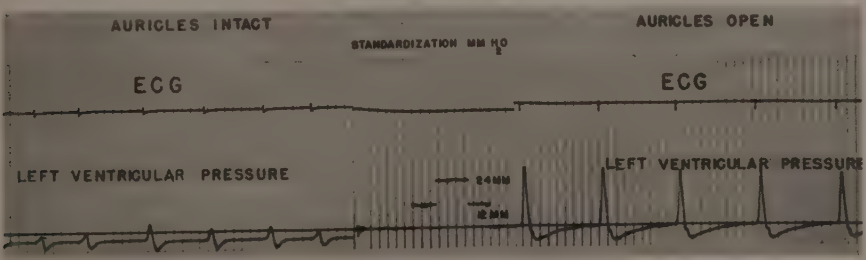

FIG. 1. Electrocardiogram and left ventricular pressure tracing on the excised beating rat heart. The continuous line in the pressure readings indicates hydrostatic zero.

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* Presented in part before Meeting of Assn. of Amer. Physicians, May, 1956.

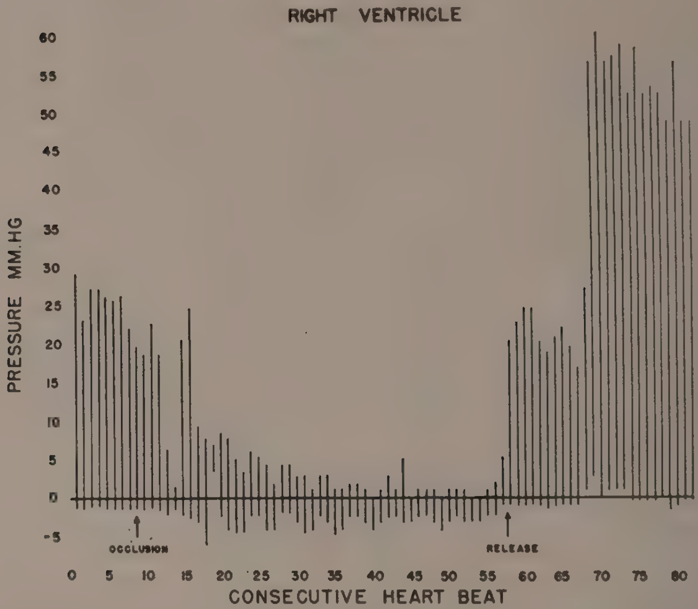

FIG. 2. Highest systolic and lowest diastolic pressure recorded from the right ventricle of the open chest dog. Pressures recorded in respect to atmospheric zero corrected for the hydrostatic column of blood above the needle. Occlusion produced by clamping superior and inferior vena cavae.

ing apparatus used in the rat studies.

Results. Simultaneous electrocardiogram and intraventricular pressure tracings of the excised, beating rat heart suspended in saline are presented in Fig. 1, first with the auricles intact and then with the auricles excised. With the auricles intact the intraventricular pressures were continuously negative, rising toward zero in systole and becoming more negative in diastole. There was no positive intraventricular pressure because there was no filling since the thin walled auricles were collapsed and fluid cannot be drawn through such a freely collapsible structure. With the auricles excised the systolic pressure in the ventricle was positive and the diastolic pressure in the ventricle slightly negative. In this preparation the maximum positive pressure during systole was similar to the maximum negative pressure in diastole with the inflow obstructed. It may be seen that the heart rate slows and the P waves of the EKG disappear when the auricles are excised.

Diastolic pressures recorded in the right ventricle of the intact dog's heart in the open chest were slightly negative and were found to become progressively more negative (–5.0 mm Hg) as the inflow (superior and inferior vena cava, coronary sinus and azygous veins) was occluded (Fig. 2). The systolic pressures also fell (from 27 mm to 2 mm) during this procedure. During each systole a positive pressure developed indicating that venous return was only partially occluded (Fig. 1). Other studies have shown that with further impairment of venous return systolic pressure was further reduced and the shape of the tracings approached that of the excised heart with intact auricles. Following release of venous occlusion the pressures in the right ventricle returned temporarily to pre-occlusion levels.

Left ventricular diastolic pressure was usually 1 to 3 mm Hg below atmospheric pressure (Fig. 3). Diminution of venous return by pulmonary venous occlusion resulted in more negative intraventricular pressures (–15 mm Hg). In other studies pressures as

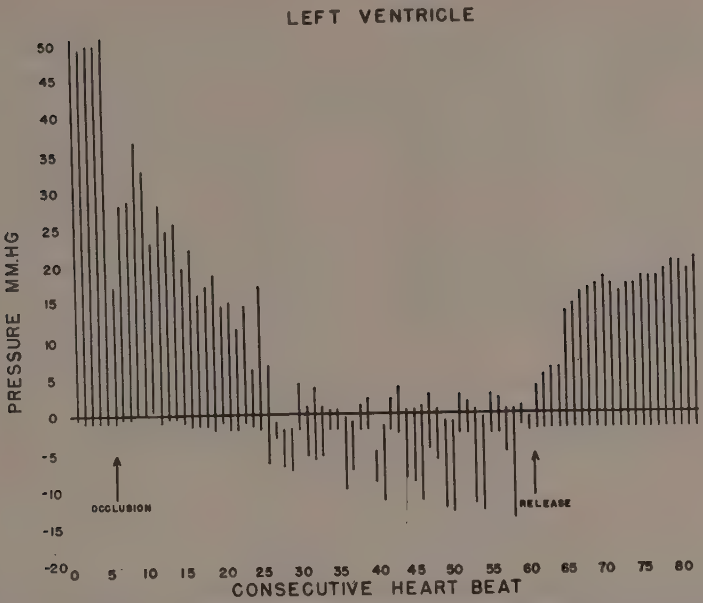

FIG. 3. Highest systolic and lowest diastolic pressure recorded from the left ventricle of the open chest dog. Pressures recorded in respect to atmospheric zero corrected for the hydrostatic column of blood above the needle. Occlusion produced by clamping pulmonary veins.

low as –25 mm Hg have been observed. Systolic pressures also fell (from 50 mm to 3 mm) following impairment of venous return to the ventricle. Following release of venous occlusion the pressures in the left ventricle returned toward normal (from 3 mm to 25 mm) and the diastolic pressures remained negative (–2 mm).

Discussion. The persistence of negative intraventricular pressure during both phases of the cardiac cycle in the excised heart with auricles intact indicated that after the heart empties inflow was impeded by the auricles and fluid cannot be drawn into the ventricles. This results in a continued negative pressure with systole only increasing pressure to zero as a result of contraction. Opening the auricles allows fluid to be drawn through the mitral valve producing a temporary negative diastolic pressure and filling of the ventricle. Systolic contraction of the filled ventricle resulted in a positive intraventricular pressure and systolic ejection of fluid. These pressure changes confirm the observation that the excised heart was capable of drawing fluid into the ventricles during diastole.

The pressure changes in the intact heart of the open chest dog indicated that the *in vitro* findings apply in the intact circulation. The diastolic negative pressures which develop during obstruction of venous return indicated that part of the energy of systole was dissipated during diastole. The left ventricle produced more negative pressures than the right ventricle which might be explained by the differences of thickness and elasticity in the ventricular walls. The slight negativity of pressure in the heart with normal venous return does not indicate absence of a force drawing fluid into the ventricle, but showed that the venous pool was adequate to satisfy the filling force.

The findings suggest that the heart functions as a pump, drawing fluid in during diastole and ejecting fluid in septole. The early rapid diastolic filling observed during cineangiocardiography(3) can be explained by these observations and a mechanism is provided to explain the fact that ventricular volume increases in diastole when intraventricular pressure decreases(4). Although the concept of the heart drawing fluid in during diastole is old(5,6,7) the magnitude of the negative pressure which can build up in diastole indicates that this mechanism is more important in cardiac filling than has been considered in the past.

We wish to thank Dr. Noble O. Fowler and Dr. E. Ronald Duschesne for their assistance.

Following the preparation of this paper, a communication with Dr. Gerhard Brecher of Department of Physiology, University of Ohio indicates that he has also obtained negative intraventricular pressures in the dog.

1. Harvey, William, *Leake translation by Chancey Leake*, Charles Thomas, Philadelphia, (1931).

2. Bloom, Walter L., *Am. J. Physiol.*, 1955, v183, 597.

3. Rushmer, R. F., and Crystal, D. H., *Circulation*, 1951, v4, 211.

4. Wiggers, C. J., and Katz, L. N., *Am. J. Physiol.*, 1922, v58, 439.

5. da Vinci, Leonardo, *On the Human Body*, Translations by C. D. O'Malley and J. B. de C. M. Saunders, Henry Schuman, N.Y., 1952, p93.

6. Goltz, Fr., and Gaule, J., *Pflügers Archiv.*, 1878, v17, 100.

7. Ebstein, E., *Erg. der Phys.*, 1904, v3, 130.

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Effect of Steroid Administration on Excretion of α-Ketolic Steroids and Sulfur in Cats. (22786)

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In the course of treating cats with 9α-fluorohydrocortisone and Δ^1-cortisone (prednisone), the changes in the chromatographic pattern of urinary steroids were measured. Two principal changes from the normal were encountered. (a) The α-ketols which were excreted increased only slightly, but there were changes in the proportions of several steroid metabolites. (b) It was unexpectedly found that elemental sulfur was present in large amounts in the chloroform extracts of these urines and that this interfered with the determination of α-ketols by the blue tetrazolium (B.T.) method, since elemental sulfur was found to give a positive B.T. reaction. After the separation of sulfur and steroids a significant increase in the urinary excretion of sulfur-containing compounds during steroid treatment was observed.

Methods. 9α-Fluorohydrocortisone and Δ^1-cortisone were administered subcutaneously in daily doses of 10 and 50 mg respectively.† The animals were fed diets of fresh meat which were constant during control and treatment periods. After 5 days or more of treatment, all urine was collected for 4 to 7 day periods and pooled. The pooled urine was then incubated with β-glucuronidase at pH 4.5 for 48 hours, extracted with chloroform at pH 1.0, and the chloroform extract washed briefly with 0.1 N NaOH and water. The B.T.-reacting materials were measured as described by Touchstone and Hsu(1). After the determination of total B.T.-reacting substances the remainder of the extract was subjected to paper chromatography.

Results. Fig. 1 shows three representative chromatograms. It appears that the principal metabolites in the normal cat occur in the region where H_4B, allo-H_4B, H_4A and H_4DOC appear (see legend for key to abbreviations). During the administration of 9α-fluorohydrocortisone and Δ^1-cortisone peaks can be found in the regions of H_4F, H_4E, B, A, and H_4DOC. Δ^1-Cortisone also caused excretion of three metabolites which were not found during administration of 9α-fluorohydrocortisone, and it seems that a large portion

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* Trainee, National Institute of Arthritis and Metabolic Diseases.

† We are grateful to Merck and Co., and to E. R. Squibb and Sons for supplying these steroids.

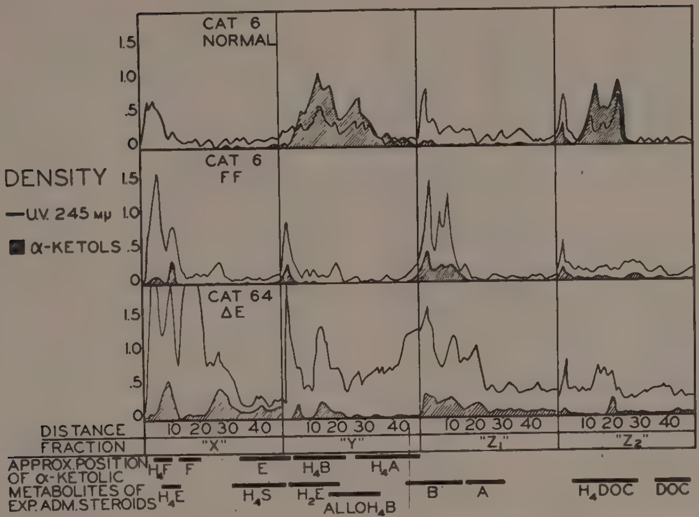

FIG. 1. Representative paper chromatograms of urinary steroid excretion in normal cat and in cats treated with 10 mg of 9α-fluorohydrocortisone (FF) daily and 50 mg of Δ^1-cortisone (ΔE) daily respectively. Chromatograms charted are based on one of eighteen ½ in. strips, each strip representing about 25% of daily steroid excretion(3). Unshaded area represents ultraviolet absorption at 245 mμ, using a Beckman spectrophotometer and special adapter for the chromatographic strips. Steroids having α,β-unsaturated ketone groupings in the A ring will be detected at this wave length, whereas compounds containing a saturated A ring (tetrahydro-forms) show no absorption. The solid silhouette represents α-ketolic compounds which reduce B.T. These were obtained by spraying strips with B.T. and measuring absorption at 600 mμ. No definite identification of the materials represented by these peaks has yet been made. On bottom of chart the approximate locations of the various metabolites in human urines chromatographed under similar conditions by Dohan, Touchstone and Richardson(3) have been drawn. H_4 = tetrahydrocortisone; H_2 = dihydrocortisone; F = hydrocortisone; E = cortisone; S = Reichstein's compound S (11-desoxy-17-hydroxycorticosterone); B = corticosterone; A = 11-dehydrocorticosterone; DOC = 11-desoxycorticosterone. Fraction "X" represents the compounds left on paper after 15-16 cc of effluent/½ in. strip had been collected in a toluene-propylene glycol system. Fraction "Y" represents rechromatography of effluent in the same system, collecting 3 cc of effluent. Fractions "Z_1" and "Z_2" both were chromatographed in methylcyclohexane-propylene glycol, collecting 15 and 5 cc of effluent respectively. Elemental sulfur, but no appreciable amount of α-ketolic steroids, was found in the last effluent "Z_2."
Distance—each 10 units = 6.5 cm on paper.

of Δ^1-cortisone is excreted as non-α-ketolic substances. By these patterns, and from histologic sections of the adrenals of the cats(2) it can be inferred that both steroids depress adrenal function.

Determinations on the initial extracts of urine with one application of B.T. yielded surprisingly high values, equalling, or even exceeding, those of man. Erratic results also indicated that the color development was slow and incomplete. By applying B.T. twice, with water washing between applications, higher and more consistent values were found. A third application resulted in no further color development. Cortisone and other steroid standards gave the same results as before. Both the single and double applications yielded a significant increase in B.T.-reacting substances after the administration of Δ^1-cortisone or 9α-fluorohydrocortisone, to the extent that in a few instances the excretion in milligrams exceeded the amount of steroid given. Further investigation showed that the bulk of B.T.-reducing material was elemental sulfur. By the double application method standard solutions of sulfur in chloroform gave a curve conforming to Beer's Law, with a slope similar to that of the steroid standards.

Fig. 2 shows the daily excretion of B.T.-positive materials in cats during control peri-

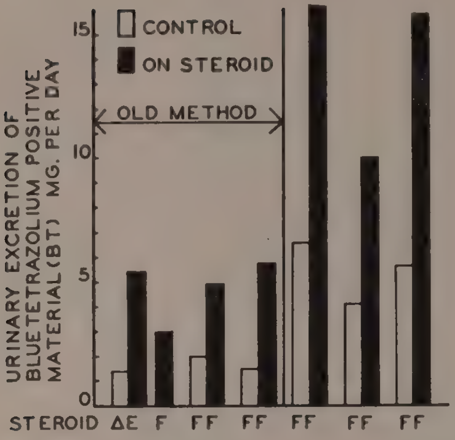

FIG. 2. Daily urinary excretion of blue tetrazolium-positive materials in cats during control periods and during steroid therapy. Old method: One application of blue tetrazolium. The 3 pairs of columns on the right show results after 2 applications of blue tetrazolium. One cat on 10 mg/day of hydrocortisone (F) showed slightly increased excretion. FF = 9α-fluorohydrocortisone.

ods and during steroid therapy. There is a consistent increase during therapy. Not charted are the amounts of B.T.-positive materials in the urine of one Houssay cat and three depancreatized animals, which were all within, or lower than, the normal range.

Fig. 3 shows the relative amounts of sulfur and steroids in extracts from which the sulfur has been separated by chromatography for 12 hours in the methylcyclohexane-propylene glycol system, by which method the less polar sulfur is found in the effluent and the α-ketolic steroids remain on the paper. The amount of steroid is small in comparison to the concentration of sulfur.

Having found that the usual extraction procedure yielded sulfur which interfered with the determination of steroids by B.T., we tried hydrolysis at pH 4.5 instead of pH 1 and found that no sulfur appeared in the extract. Evidently acid hydrolysis is an important determinant in liberating elemental sulfur.

Discussion. These questions now arise: What is the origin of the sulfur, and what is the significance of the increase in sulfur excretion during steroid administration?

Westall(4) reported that cats excrete in the urine large amounts of a peptide, which he called felinine. This compound has been tentatively found to consist of a combination of cysteine and isoamyl alcohol, combined in a thioester linkage. However, extraction of cysteine under the conditions used here failed to show any decomposition to sulfur. The diet does not seem to account for the increase in sulfur excretion, as the cats were fed the same amount of meat before and during therapy.

Negative nitrogen balance *per se,* as demonstrated in the Houssay animal and three depancreatized cats, does not appear to produce an increase in the sulfur excretion. Neither does the presence of glycosuria have an influence, as some steroid treated cats were aglycosuric during the collection period. We have observed that animals treated with steroids have no hair growth, and that the skin is thin and friable. In one cat, gentle handling caused the skin to rupture. There is also severe wasting of muscle, with profound muscle weakness. Both hair, epidermis and muscle are rich in sulfur-containing substances. It is possible that the steroids upset normal metabolism of certain sulfur-containing compounds in these tissues. However, by these methods we have not found sulfur in the urine of man during doses of prednisone up to 80 mg daily.

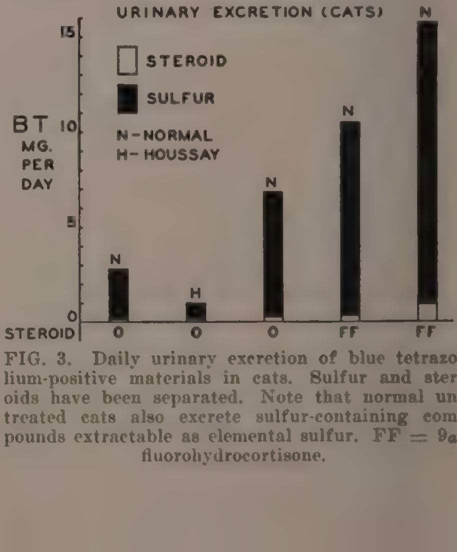

FIG. 3. Daily urinary excretion of blue tetrazolium-positive materials in cats. Sulfur and steroids have been separated. Note that normal untreated cats also excrete sulfur-containing compounds extractable as elemental sulfur. FF = 9α-fluorohydrocortisone.

Summary. 1. Administration of Δ^1-cortisone and 9α-fluorohydrocortisone changes normal urinary excretion of steroid metabolites, indicating adrenal depression or altered steroid metabolism. 2. Only a small proportion of administered 9α-fluorohydrocortisone is excreted as α-ketolic metabolites; Δ^1-cortisone also is converted to a great extent to non-α-ketolic elements. 3. Elemental sulfur has been found to give a positive reaction with blue tetrazolium. This interferes with the quantitative determination of α-ketols in extracts of cat urines. 4. The α-ketols and sulfur can be separated by chromatography and each determined quantitatively by the blue tetrazolium reaction, using a modified method. 5. Administration of Δ^1E and 9α-fluorohydrocortisone causes an increase in urinary excretion of sulfur-containing compounds in cats, which leads us to conclude that these steroids alter sulfur metabolism.

1. Touchstone, J. C., and Hsu, Chien-Tien, *Anal. Chem.*, 1955, v27, 1517.

2. Buse, J. F., Jr., Gundersen, K., and Lukens, F. D. W., *Diabetes.*

3. Dohan, F. C., Touchstone, J. C., and Richardson, E. M., *J. Clin. Inv.*, 1955, v34, 485-99.

4. Westall, R. G., *Biochem. J.*, 1953, v55, 244.

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Prostatic Neoplasms of the Wistar Rat Induced with 20 - Methylcholanthrene. (22787)

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Prostatic tumors have never been reported to occur spontaneously in the rat, in contrast to man(1) and dog(2,3). McCoy(4) examined nearly 100,000 wild rats finding 103 primary tumors, none of which originated in the prostate. Moore and Melchionna(5) were first to induce with 1:2 benzpyrene in lard squamous cell carcinoma and sarcoma of the anterior lobe of the rat prostate but they did not report metastases. Dunning, Curtis and Segaloff(6) produced metastasizing squamous cell carcinoma in rat prostate with methylcholanthrene but the designation of the prostatic lobe where this carcinoma was induced was not mentioned. Neither groups reported occurrence of adenocarcinoma of the rat prostate.

The purpose of this paper is to report the induction of three types of experimental neoplasms particularly adenocarcinomas in the ventral lobe of the prostate glands of Wistar rats using methylcholanthrene crystals.

Material and methods. 122 male inbred Wistar rats, 3 months of age, were kept in individual cages and given Purina Laboratory chow and water *ad libitum*. Under nembutal or ether anesthesia, the ventral prostate was exposed through a posterior midline incision. Using aseptic technic, a pocket was made in the ventral prostate, and crystals of 20-methylcholanthrene were carefully deposited. The pocket was then closed by suturing with surgical silk. Extreme care was taken to avoid scattering of methylcholanthrene. The technical success of the intraprostatic injections was evidenced by presence of a small yellow bleb. Following the operation, the rats were examined routinely for presence of palpable prostatic masses; biopsies were repeatedly performed to study the character of the growth. This method was used to establish the latent period of the tumor. 23 animals were excluded from evaluation because they died shortly after the operation. The remainder of operated rats, 99 in number, were kept until they showed signs of impending death. These were sacrificed and autopsied. Wherever possible, small fragments of prostatic tumors were removed and immedi-

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TABLE I. Induced Prostatic Neoplasms in 99 Intact Wistar Rats.

Tumor latent period (days)	Adeno-carcinoma	Squamous cell carcinoma	Leiomyo-sarcoma
100-150		1	
151-200	2	19	
201-250		4	
251-300		2	
301-350		4	3*
Total	2 (2%)	30 (30.3%)	3 (3%)

* 2 rats having leiomyosarcoma had associated squamous cell carcinoma.

ately inoculated subcutaneously into rats of the same strain. The tissue for histologic study was fixed in formaldehyde or Bouin's fluid, embedded in paraffin and sections stained with Harris' hematoxylin and eosin.

Results A. Tumor incidence, latent period and growth behavior of prostatic neoplasms. Table I shows the incidence and latent period of various prostatic neoplasms. Fig. 1 shows a section of a normal mature 6-month-old rat prostate and Fig. 2-5 illustrate prostatic tumors observed in experimental rats. The earliest squamous cell carcinoma was seen on 118th day. Adenocarcinomas appeared on 180th and 198th day. Leiomyosarcoma of the prostate occurred after 301 days, much later than any of the other neoplasms. In a different series 24 rats that had their ventral prostates sutured only with surgical silk had not developed neoplasms. The average dimensions of the tumors were 3.5 x 4 x 2.5 cm. The squamous cell carcinoma grew the fastest. The average survival time beyond the latent period of rats bearing these tumors was 63 days for squamous cell carcinomas, 92 days for leiomyosarcomas, and 63 days for adenocarcinomas. Death was due to obstruction and/or metastases produced by the tumor. Hydronephrosis, hydroureter, bladder obstruction, anemia and hematuria were complications often seen in rats in which the prostatic masses grew to 3 x 2.5 x 1.5 cm or more. Near death the rats became very irritable, and external and internal bleeding was evident.

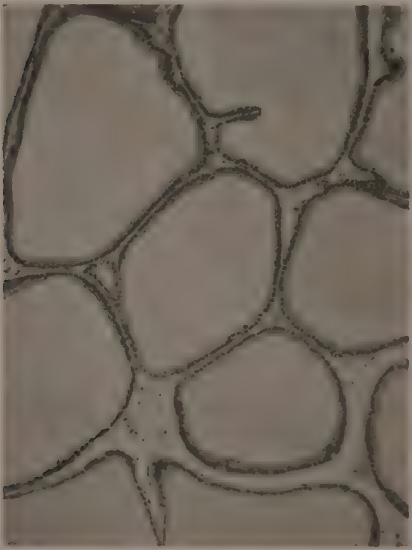

FIG. 1. Section through normal mature 6 mo old rat prostate, ventral lobe. Mag. 150× H & E.

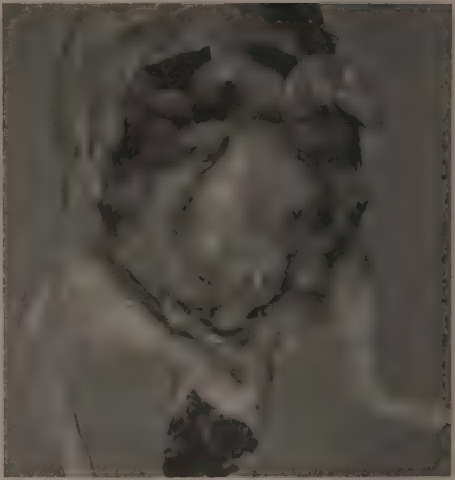

FIG. 2. Photograph of Wistar rat bearing an *in situ* adenocarcinoma of prostate gland. Tumor was 2.8 × 3.2 × 2.5 cm and metastasized to lungs and ribs. Tumor latent period 180 days.

Fragments of 21 of the squamous cell carcinomas were transplanted into rats of the same strain. Table III summarizes the growth behavior of 4 of these tumors. Unfortunately, the adenocarcinomas and leiomyosarcomas were not transplanted. Detailed data in Table III on the growth behavior of these transplantable tumors at various generations are advanced to illustrate

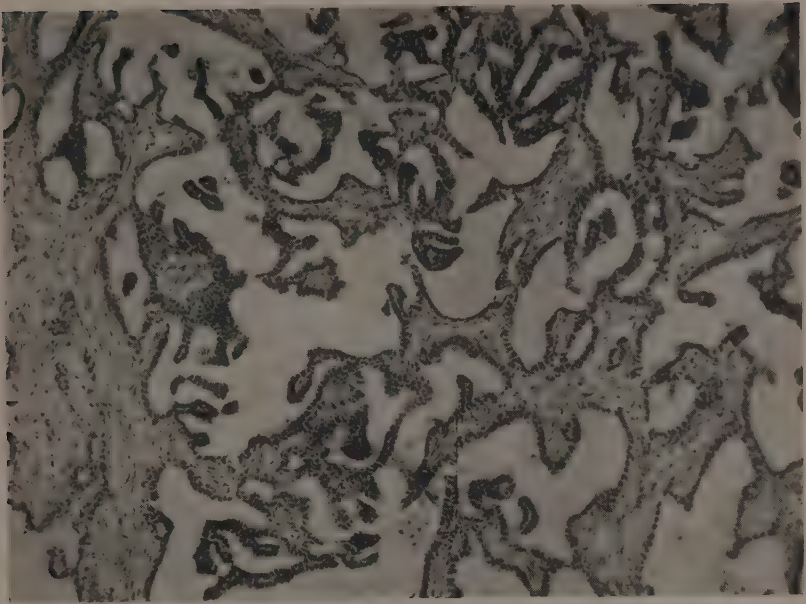

FIG. 3. Section through adenocarcinoma of rat prostate. Sections through tumor mass revealed that there was diffuse infiltration by adenocarcinoma. Carcinomatous acini show characteristic lack of orientation. In some area there was a lack of basement membrane encircling each gland, and irregularity of glandular epithelium. No presence of secretion in the gland was seen. Mag. 150× H & E.

similarities and differences that exist among the 4 tumors. Some of the prostatic tumors that metastasized grew very poorly or not at all in inbred female rats, although the reverse was true when transplanted to male rats.

Histological Findings B. — Of the 35 tumors examined histologically on the basis of cellular structure of the tumor and its staining properties, two were judged to be adenocarcinomas, 30 squamous cell carcinomas, 3 leiomyosarcomas (Fig. 2-5). Invasion and/or metastases of these tumors are summarized in Table II. In most instances, prior to appearance of neoplasms, considerable inflammatory reactions, squamous cell metaplasia and abscess formation were observed, and these reactions were frequently seen in rats that developed squamous cell carcinoma and leiomyosarcoma, but not in rats developing adenocarcinoma of the prostate.

In the 2 adenocarcinomas examined, the tumor cells were arranged in glandular formation around irregular spaces, or trabeculae and the stroma was composed of a small

TABLE II. Frequency Distribution of Metastases and/or Invasion of Induced Prostatic Neoplasms in Wistar Rats. Leiomyosarcoma did not metastasize.

Organ	Squamous cell carcinoma	Adeno-carcinoma
Lung	10	1
Thymus	1	0
Heart	1	0
Liver	3	0
Spleen	1	0
Pancreas	1	0
Adrenal	3	0
Kidney	4	0
Lymph nodes	8	0
Intestines	5	0
Seminal vesicle	12	0
Coagulating gland	12	0
Dorsal prostate	14	0
Testes	2	0
Epididymis	6	0
Ribs	2	1
Pelvic bone	0	0
Bladder	11	1
Diaphragm	3	0

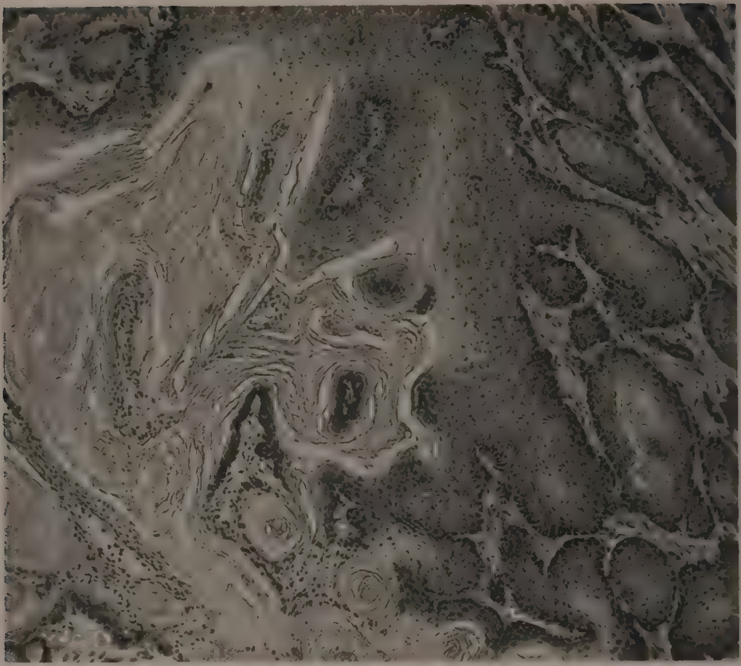

FIG. 4. Section through squamous cell carcinoma of rat prostate. Tumor measured was 3.9 × 4.8 × 2.2 cm and metastasized to lung, rib, kidney, mesentery, and omentum. Mag. 150× H & E.

amount of connective tissue accompanied by slender capillaries. The tumor cells had round or oval hyperchromatic nuclei of varying size situated in the basal part of the cells. The cytoplasm appeared more swollen and foamy or vacuolated and the nucleus became pyknotic. No evidence of secretion was seen in the gland with diffuse infiltration by adenocarcinoma.

The leiomyosarcomas consisted of cells that were large and spindle-shaped with abundant eosinophilic cytoplasm; among these were many giant cells. In some areas of the tumors, the cells showed pleomorphism with round or oblong nuclei as the predominant form and many mitotic figures were common. The cells of this tumor often tended to form interlacing bundle running in different directions. Two of the three rats with leiomyosarcomas that had a latent period of 301 and 308 days respectively had associated with it areas of squamous cell carcinoma. Although the leiomyosarcomas did not metastasize, the general histological appearance supported the belief that the tumors were malignant.

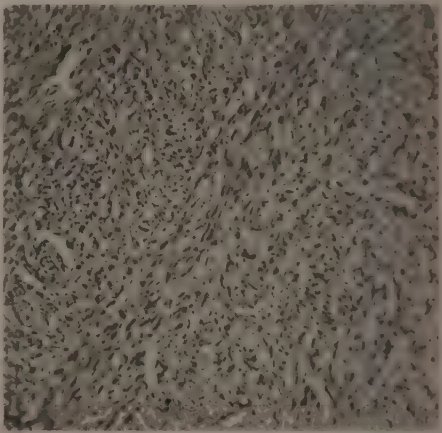

FIG. 5. Section through rat prostate with leiomyosarcoma. Tumor measured 2.8 × 2.2 × 3.0 cm, grew slowly and did not metastasize. Glandular nature of prostate was completely absent. Mag. 125× H & E.

TABLE III. Results of Homologous Transplantation of Several Prostatic Squamous Cell Carcinomas into Wistar Rats.

Designation No. of tumor	Response of tumor at generation	No. of rats transplanted into	Site in host inoculated	Growth characteristics			Infiltration and metastases	Special features
				Takes, %	Latent periods, days	Kill host, days		
1	12	12 ♂	Subcut.	90	8–10	30–40	Local invasion, lung	No regression, ulceration
2	18	12 ♂	”	60	14–21	30–60	Local invasion	Regression after ulceration (15%)
		12 ♀	”	70	12–18	25–52	*Idem*	” ” (20%)
3	16	12 ♂	”	85	7–14	25–30	Lung, lymphnodes	Hemorrhage, necrosis, no regression
		12 ♀	”	20	14–22	40–56		Hemorrhage, necrosis, regression
		12 ♂	Intraper.	56		44–54	Lung, ribs, local invasion	Necrosis, hemorrhage, no regression
		12 ♀	”	0				
		12 ♂	Pleura cavity	36		18–23	Lungs, ribs, adrenal diaphragm	Necrosis (less extensive), hemorrhage, no regression
		12 ♀	*Idem*	0				
4	10	12 ♂	Subcut.	25	14–28	50–60	Lung, occasional local invasion	Regression rare, necrosis (less extensive)
		12 ♀	”	10	24–28			Regression

Histologically most squamous cell carcinomas were composed of irregular masses or sheets of more or less differentiated basal and columnar cells with an inconspicuous stroma. In many of the tumors, the cancerous cells formed epithelial pearls or small cystic areas with mucoid material in the center. In some tumors the epithelial elements which differentiated and formed keratin, also had areas that appeared glandular. It seemed as if the "neoplastic" change did not go on to full squamous cell differentiation.

Discussion. It has been shown that prostatic neoplasms, similar to the 3 types that occur in man, can be induced with methylcholanthrene in the ventral prostate of Wistar rats, squamous cell carcinoma being more frequent in the rat than in man. Adenocarcinoma of the prostate gland in the rat showed great similarity to that of man. Moreover, these neoplasms with the exception of leiomyosarcoma not only exhibited extensive local growth and invasion, but also distant metastases. Judging by distribution of metastases in various organs, the tumors were autonomous, and spread was through blood and/or lymphatic pathways.

The fact that this non-specific, poorly soluble carcinogen induced so high a proportion of prostatic squamous cell carcinomas proved not to be out of keeping with the results of Moore *et al.*(5) and Dunning *et al.*(6). On the other hand, our results differ from the above investigators by the induction of adenocarcinomas and leiomyosarcomas, by a more extensive distribution of invasion and/or metastases of prostatic tumors to various organs, and by a more detailed account on the growth characteristics of transplantable prostatic tumors, some of which are in their 18th transplantable generation. Moore *et al.* and Dunning *et al.* used a different strain or subline of rats in their respective studies. It is noteworthy to point out that different species and sometimes even different strains of the same species vary markedly in the relative sensitiveness of their various tissues to induced and spontaneous tumor development. Moreover, the nutritional, hormonal and metabolic status of the gland are important factors too, that can greatly affect the tumorgenic response of a gland to a carcinogen.

It is becoming increasingly important that when working with the prostatic gland of the rat that the designation of the prostatic lobes be carefully indicated despite having a common origin arising as buds of the urogenital sinus. The observation that the prostatic lobes in an animal is not a homogenous structure as judged by its response to estrogen, enzyme concentration, chemical constituents (7) and zinc^{65} uptake(8,9) has been late in discovery notwithstanding the fact that histological procedures do not reveal any differences among the lobes of the prostate gland. It is therefore reasonable to assume that lobes of the rat prostate gland can react differently to methylcholanthrene or to other carcinogens. Preliminary studies using different carcinogens to various prostatic lobes seem to support this concept.

Summary. (1) Intraprostatic deposits of 20-methylcholanthrene crystals induced three types of prostatic neoplasms in the ventral lobes of the Wistar rat's prostate gland: adenocarcinoma (2%), leiomyosarcomas (3%) and squamous cell carcinomas (30.3%). The squamous cell carcinomas and adenocarcinomas metastasized. (2) A detailed account of the distribution of invasion and/or metastases of these prostatic tumors to various organs are presented as well as an account on the growth characteristics of transplantable squamous cell carcinomas.

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1. Huggins, C., *Harvey Lectures,* 1947, v42, 148.
2. Folger, A. F., *Ergebn. d. allg. Path. u. path. Anat.,* 1917, v18, 372.
3. Feldman, W. H., *Neoplasms of Domesticated Animals, Mayo Clinic Monographs, Philadelphia,* 1932, W. B. Saunders Co.
4. McCoy, G. W., *J. Med. Research,* 1909, v16, 285.
5. Moore, R. A., and Melchionna, R. H., *Am. J. Cancer,* 1937, v30, 731.
6. Dunning, W. F., Curtis, M. R., and Segaloff, A., *Cancer Research,* 1946, v6, 256.
7. Huggins, C., and Webster, W. C., *J. Urol.,* 1948, v59, 258.
8. Gunn, S. A., and Gould, T. C., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 17.
9. Mirand, E. A., and Bender, M., *Anat. Rec.,* 1956, v125, 618.

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Nitrogen Sparing Action of Neutral Fat and Fatty Acids in the Phlorhizinized Rat.* (22788)

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In a recent review devoted to the relationship of protein metabolism to that of fat and carbohydrate, Munro summarized the extensive data indicating that carbohydrate is a protein sparer, regardless of whether the animal is starving or is receiving a protein free or protein containing diet(1). By contrast, Munro noted that the results of studies dealing with the ability of fat to prevent excessive nitrogen loss are in conflict. Thus, some reports indicate that when fat is the sole source of calories, the nitrogen output of an animal is as great as during total starvation(2,3,4,5); on the other hand, data has been presented which demonstrates that fat is a protein sparer, even in the absence of protein in the diet, if minimal caloric requirements are satisfied(6,7). In view of the divergent results, and because it seemed to us that fat, under

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TABLE I. Composition of Diets.

Vitamins and salt

Salt (Wesson)	20 g	Vit. B syrup	30 ml
Cod liver oil	5 ml	2-methyl naphthoquinone	50 mg
Wheat germ oil	5 "		

To the above were added, for the respective diets:

	High carbohydrate	Moderate carbohydrate	High fat
	g		
Casein hydrolysate (enzymatic)	95	95	95
Lactalbumin hydrolysate (enzymatic)	10	10	10
Methionine	2	2	2
Liver powder	10	10	10
Corn starch	185	110	
Dextrin	170	105	
Sucrose	170	110	
Mazola oil	10	100	245
Gum ghatti			10

Solid diet: To moderate carbohydrate diet were added 300 ml of 5% agar agar and 300 ml of water.

Liquid diets: To appropriate diet were added 30 g of cellu flour and water to make 1000 ml.

Protein-free diets: Made up similar to liquid diets with exception that casein and lactalbumin hydrolysates, methionine and liver powder were omitted.

Oleic acid diet: To vitamins and salt, 30 g of cellu flour, 10 g of gum ghatti, 240 g of oleic acid and water to make 1000 ml were added.

Glycerol diet: To vitamins and salt, 30 g of cellu flour, 10 g of gum ghatti, 25 g of glycerol and water to make 1000 ml were added.

appropriate conditions, should spare protein in the phlorhizinized rat, the experiments described below were undertaken. Protein sparing effects were obtained in phlorhizinized rats tube fed protein free diets, irrespective of whether the calories were supplied by neutral fat, fatty acids or carbohydrate.

Methods. Male Holtzman rats, weighing approximately 120 g at the start of the experiment, were employed and fed diets modified from those described by Ingle(8). The food was available to the animals eating *ad libitum* in solid form at all times but was administered by stomach tube to the force fed groups in liquid state twice a day, before 9 a.m. and after 4 p.m. The compositions of the diets are given in Table I. Four series of rats were employed: (1) Group 1 was *ad libitum* fed the solid moderate carbohydrate diet; (2) Group 2 was force fed the moderate carbohydrate diet; (3) Group 3 was force fed the high carbohydrate diet; (4) Group 4 was force fed the high fat diet. The rats were placed on their respective diets for 3-4 weeks, until they weighed between 210 and 220 g, the amount of diet administered to the rats in the force fed series being adjusted so that the weight gains of these animals approximated that of the *ad libitum* fed ones. At the completion of the feeding period, the rats were injected intramuscularly on each of 2 successive days with 50 mg of phlorhizin in 1 ml of sesame oil. The dietary management of the animals during the 2 days is shown in Table II. All animals were placed in individual metabolism cages during the second day of phlorhizinization and their urines collected under toluene with a gram of citric acid as an acidifying agent. These 24 hour specimens

TABLE II. Dietary Management of the Rats during the Two Days of Phlorhizinization.

Rat group		Period of starvation		Protein-free diets fed*				
		Day 1	Day 2	Moderate carbohydrate	High carbohydrate	High fat	Oleic acid	Glycerol
1†		+	+	0	0	0	0	0
2‡	(a)	0	0	+	0	0	0	0
	(b)	+	0	+	0	0	0	0
3§		0	0	0	+	0	0	0
4‖	(a)	0	0	0	0	+	0	0
	(b)	+	0	0	0	+	0	0
	(c)	+	0	0	0	0	+	0
	(d)	+	0	0	0	0	0	+

* Diet force fed the day(s) the animals were not starved. † These rats previously were on *ad lib* diet. ‡ Had been previously force fed the moderate carbohydrate diet. § High carbohydrate diet. ‖ High fat diet.

+ = Treatment indicated; 0 = no treatment given.

TABLE III. Excretion of Nitrogen and Glucose after Injections of Phlorhizin.

Rat group†		N excreted*	Glucose excreted*
		mg/100 g body wt	
1‡	(7)	125	331
		109–156	233– 429
2§ (a)	(6)	42	946
		39– 54	743–1220
3‖	(7)	35	1053
		28– 41	790–1305
4¶ (a)	(5)	67	445
		54– 88	413– 500

* Values given are mean excretions and ranges for the group.

† No. in parentheses refers to No. of animals in group.

‡ These rats had previously been fed *ad lib*; they were starved for 2 days of phlorhizinization.

§ These rats were force fed the moderate carbohydrate protein free diet for 2 days of phlorhizinization.

‖ Force fed high carbohydrate protein free diet for 2 days of phlorhizinization.

¶ Force fed high fat protein free diet for 2 days of phlorhizinization.

were analyzed for N.P.N. by a modified Conway technic(9) and for reducing substances by the method of Nelson(10).

Results. Table III shows the urinary outputs of N.P.N. and glucose for the second day in rats force fed protein free diets during the 2 days of phlorhizinization, as well as the urinary losses of the starving animals. It can be seen that the N.P.N. output dropped from 125 mg/100 g in the animals who did not eat to approximately 40 mg/100 g in the animals force fed the carbohydrate diets. Little difference can be noted between the nitrogen excretions of the animals receiving the high or moderate carbohydrate intakes. When fat in the form of mazola oil was the source of calories, the nitrogen loss fell to approximately 67 mg/100 g of rat.

Starvation during the first day of phlorhizinization, followed by the feeding of the respective protein free diets on the second day, resulted in outputs similar to those noted above (Table IV). Fat feeding, in the absence of protein or carbohydrate, appeared to decrease the urinary nitrogen excretions.

TABLE IV. Excretion of Nitrogen and Glucose after Injections of Phlorhizin.

Rat group†		N excreted*	Glucose excreted*
		mg/100 g body wt	
1‡	(7)	125	331
		109–156	233– 429
2§ (b)	(5)	40	1126
		37– 43	803–1394
4‖ (b)	(6)	59	463
		43– 75	176– 947
(c)	(5)	62	368
		55– 81	211– 424
(d)	(6)	47	482
		38– 57	404– 634

* Values given are mean excretions and ranges for the group.

† No. in parentheses refers to No. of animals in group.

‡ These rats had previously been fed *ad lib*; they were starved for 2 days of phlorhizinization.

§ These rats were starved for the first and force fed the moderate carbohydrate protein free diet for second day of phlorhizinization.

‖ These rats were starved for first day of phlorhizinization; on second day, rats of groups (b), (c) and (d) were force fed protein free high fat, oleic acid and glycerol diets respectively.

The D:N ratios obtained in the above experiments suggested that gluconeogenesis from fat was occurring. However, since the dietary source of fat (mazola oil) is at least 90% trioleate[‡] and therefore contains 10% available glycerol when hydrolyzed, it became apparent that both the "gluconeogenesis" and protein sparing we were observing might be attributable to the glycerol moiety of the fat. Accordingly, two groups of rats who had been force fed the high fat diet for 3-4 weeks were phlorhizinized for two days, starved on the first day but on the second day were force fed oleic acid or glycerol in the amounts calculated to be present in the high fat (mazola oil) diet. From the results in Table IV, it may be seen that the oleic acid, in the absence of glycerol or any other form of carbohydrate, prevented the excessive protein catabolism that results from phlorhizinization.

Discussion. The results obtained in our phlorhizinized rats, fed isocaloric amounts of protein free diets containing large or moderate amounts of carbohydrate or large amounts of neutral fat or fatty acids indicate that fat can inhibit endogenous protein catabolism. The degree to which fat prevents protein breakdown was not so great as that observed with carbohydrate; with the latter diets, N.P.N. excretions were decreased by 68%, with mazola oil by 53% and with oleic acid by 50%. Glycerol, in amounts of only 500 mg per rat per day decreased N.P.N. outputs by 63%.

[‡] We are indebted to Corn Products Refining Co. for this information.

The fats usually employed in laboratory diets are essentially triglycerides and contain 10% glycerol in ester linkage. In a high fat diet, as much as 500 mg/day of glycerol may come from this source. Failure to appreciate that 10% of dietary fat becomes available as carbohydrate when metabolized may be a factor in explaining the conflicting results obtained in previous studies.

Our first experiments were performed on animals force fed their respective protein free diets during the 2 days of phlorhizinization. To rule out the possibility that the apparent sparing of protein by fat may have been associated with the force feeding on the first day of phlorhizinization, thereby conserving body carbohydrate and protein stores, observations were made on animals starved for the first day of phlorhizin injections. On the second day, the animals were given their respective protein free diets or the oleic acid or glycerol diets (Table II). As is evident from Table IV, carbohydrate, neutral fat or fatty acids still prevented the usual protein loss seen with phlorhizinization.

While our findings are in accord with previously published data on the nitrogen sparing action resulting from feeding carbohydrate to phlorhizinized animals, they do not agree in respect to fat feeding. It has been reported that when fat, in the absence of protein, is administered to phlorhizinized animals, the accelerated nitrogen loss continued unabated (11,12,13). In contrast to previous work, we find a marked decrease in N.P.N. excretion when a protein free diet containing neutral fat or fatty acids is administered. The difference between our results and those of others may possibly be attributed to the pre-experimental dietary history of the animals; our rats were adapted and maintained on a high fat diet for 3-4 weeks before they were phlorhizinized whereas previous workers did not so adapt their animals. Munro is of the opinion that for the nitrogen sparing action of fat to be seen, studies should be performed several days after the animals have been placed on the high fat regimen(14).

It is not surprising that fat, like carbohydrate, should be found to spare nitrogen. Protein, carbohydrate and fat are considered to share a common final metabolic pathway with respect to carbon dioxide production (and energy yielding reactions). When there is a demand for energy, carbohydrate is probably utilized preferentially to protein. The findings in the carbohydrate fed phlorhizinized rats are in accord with this reasoning. On the basis of our results in the animals fed fatty acids during phlorhizinization, it appears that fat likewise is utilized in preference to endogenous protein for energy yielding reactions. However, since fat is not thought to be converted to carbohydrate, the use of protein for carbohydrate formation should cause fat fed phlorhizinized animals to excrete more nitrogen than carbohydrate fed ones. Our results are in agreement with this possibility since the N.P.N. excretions of the animals fed fat were slightly greater than the excretions of the ones fed carbohydrate.

Summary. Rats were adapted to the force feeding of a moderate or high carbohydrate or high fat diet for several weeks before phlorhizinization. During the second day of phlorhizin injections, the animals were fed their respective diets, rendered protein free, and the urinary excretions of N.P.N. measured and compared to the outputs of starving animals. The feeding of either neutral fat or carbohydrate reduced N.P.N. losses. A similar sparing of endogenous protein occurred when animals, fed the high fat diet for 3 weeks, were given oleic acid, rather than mazola oil, on the second day of phlorhizinization.

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1. Munro, H. N., *Physiol. Rev.*, 1951, v31, 449.

2. Bartman, A., *Z. f. Biol.*, 1912, v58, 375.

3. Schreiber, M., and Elvehjem, C. A., *J. Nutrition*, 1955, v57, 133.

4. Rosenthal, H. L., *ibid.*, 1952, v48, 243.

5. Munro, H. N., and Naismith, D. J., *Biochem. J.*, 1953, v54, 191.

6. Bosshardt, D. K., Paul, W. J., O'Doherty, K., and Barnes, R. H., *J. Nutrition,* 1948, v36, 773.

7. Swanson, P., and Clark, H. E., *Ann. Rev. Biochem.,* 1950, v19, 235.

8. Ingle, D. J., and Nezamis, J. E., *Am. J. Physiol,* 1950, v162, 1.

9. Conway, E. J., *In Microdiffusion Analysis and Volumetric Error, London,* Crosby, Lockwood, 1950.

10. Nelson, N., *J. Biol. Chem.,* 1944, v153, 375.

11. Takao, T., *Biochem. Z.,* 1926, v172, 272.

12. Wierzuchowski, M., *J. Biol. Chem.,* 1927, v73, 417.

13. McKee, F. W., and Hawkins, W. B., *Physiol. Rev.,* 1945, v25, 255.

14. Thomson, W. S. T., and Munro, H. N., *J. Nutrition,* 1955, v56, 139.

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Ergotamine, Oxytocin and Milk Let-Down in Lactating Rat.* (22789)

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It has been demonstrated(1-4) that adrenalin in appropriate doses can block the milk let-down response to both endogenous and injected oxytocin. Adrenalin presumably acts by constricting mammary blood vessels thus blocking passage of oxytocin to mammary myoepithelium(5-6). Ergotamine, also a peripheral vasoconstrictor(7), has recently been shown to inhibit milk let-down in lactating rats(8). The present paper is concerned with effects of exogenous oxytocin on ergotamine-inhibition of milk let-down in lactating rats. Evidence is presented to indicate that ergotamine exerts its inhibition by means other than through vasoconstrictive effects.

Materials and methods. Docile lactating albino rats, each with its first litter and weighing 200-260 g, were housed in individual cages and given free access to food and water. Each litter was reduced to 8 young shortly after birth and, whenever possible, these were equally distributed as to sex. Rats with less than 8 young were discarded. Each of 42 litters when 14 days old was isolated from their mother for 10 hours. During this time they were kept warm to prevent any undesirable effects from exposure. Each litter was then divided equally. One half was weighed as a group, killed and their stomachs examined for the presence of milk. The other half was replaced with the mother and allowed to suck for exactly 20 minutes. Length of time before each mother began nursing was recorded. Young were then removed, weighed as a group and immediately killed by decapitation. Their stomachs were opened and semisolid curds removed and weighed (Table I). The mothers received single subcutaneous injections as follows: (a) 17 received 1 mg/kg ergotamine tartrate.‡ Of these 10 were injected 10 minutes, the rest, 2 minutes before replacement of litters. (b) 10 received 1 mg/kg ergotamine 10 minutes before replacement of litters followed by oxytocin ('Pitocin', Parke Davis & Co.) 8 minutes later. Oxytocin was given to 5 rats in dose of .4 I.U./kg and to remaining 5 in dose of .2 I.U./kg. (c) 5 were treated with .2 I.U./kg oxytocin 10 minutes before replacement of litters followed by 1 mg/kg ergotamine 8 minutes later. (d) 10 served as controls of which 5 were uninjected. The remaining received comparable injections of distilled water 10 minutes prior to replacement of litters. Topical effects of saline solutions of 1:2000 ergotamine and .2 I.U./ml oxytocin alone, and in combination, were studied in 5 lactating rats. Each mother was separated 4-6 hours from its litter on the 14th day postpartum. Nembutal anaesthesia (3 mg/

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† Postdoctoral Fellow of the National Institutes of Health.

‡ Kindly supplied by Sandoz Chemicals, N. Y.

TABLE I. Average Quantity of Milk, Expressed as % Litter Body Weight, Secured in 20 Min. by Litters, Each with 4 Young, on the 14th Day Postpartum.

Treatment	No. of rats	Time of treatment prior to nursing (min.)	Avg wt of litters (g)	Avg wt of milk (g)	% milk / Litter wt	S.E.
Control	10	10	89.2	3.3	3.7	.43
Ergotamine, 1 mg/kg	10	10	86.8	.6	.7	.21
Idem	7	2	94.0	2.0	2.1	.68
Ergotamine, 1 mg/kg, then oxytocin, .2-.4 IU/kg	10	10 2	96.9	4.2	4.3	.31
Oxytocin, .2 IU/kg, then ergotamine, 1 mg/kg	5	10 2	97.7	4.2	4.3	.51

100 g) was injected intraperitoneally, an abdominal mammary gland exposed and observed with a stereoscopic dissecting microscope (x16-x32). The exposed tissue was kept moist with physiological saline solution and covered with a thin sheet of polyethylene. Drugs were administered as drops at the tip of a 24 gauge needle and gently placed on mammary tissue without touching it with the needle.

Results. At the end of 10 hours isolation, stomachs of half of each litter contained no milk. It was assumed, therefore, that stomachs of other half of each litter permitted to suck following isolation, were also empty prior to sucking. In each case upon placing the litter back with their mother after isolation, she was observed to hastily gather her young and to commence nursing within 3 minutes.

Amount of milk obtained by litters of lactating rats injected with ergotamine 10 minutes prior to litter replacement was very small in comparison with that obtained by control offspring ($P = .001$), or with that obtained by young of mothers treated with ergotamine 2 minutes before litter replacement ($P = .03$). This latter group obtained an average of only 57% as much as control offspring though the quantity difference was not significant ($P = .06$). When oxytocin was injected before or after ergotamine insignificantly larger ($P = .4$) quantities of milk were obtained by offspring in comparison with control group. The amount was, however, significantly greater than that obtained by litters of rats treated with ergotamine either 10 or 2 minutes prior to replacement of young. ($P = .001$ and $.005$ respectively). There was little difference in amount of milk obtained by offspring of uninjected and distilled water-injected controls.

Topical administration of ergotamine to living rat mammary tissue resulted in constriction of arterioles but not of venules or of alveolar capillaries. Constriction occurred within 1-10 minutes with relaxation taking place 3-15 minutes later, depending upon vessel diameter. Effect on mammary alveoli and ducts varied from no response, in most instances, to slight transient contraction of a few alveoli. Oxytocin administered alone caused almost immediate (2-4 sec.) contraction of alveoli which resulted in ducts filling with milk but there was no apparent effect on the vascular bed. If the glands were not engorged, milk did not return to the alveoli, which remained contracted. If alveoli were greatly distended, milk squeezed into ducts gradually ran back into alveoli again. Such relaxation occurred within 1-5 minutes. It was possible, therefore, in some cases to apply ergotamine and/or oxytocin to same area of tissue and obtain another contractile response as soon as relaxation of alveoli and arterioles occurred.

Ergotamine and oxytocin mixed together and applied topically, evoked both alveolar contraction and arteriole constriction. Moreover, oxytocin applied at height of ergotamine-induced arteriole constriction, elicited its usual alveolar contraction with no apparent delay in contraction time. Drops of saline applied directly to mammary tissue had no apparent effects.

Discussion. These observations would

seem to rule out ergotamine inhibiting milk let-down in lactating rats by vasoconstriction of mammary vessels. There remain at least two other possibilities to explain this inhibition. It is generally accepted(9,10) that mammary myoepithelial cells are the contractile elements responsible for squeezing milk from alveoli and ducts. There may then be a competition between oxytocin and ergotamine at the myoepithelium level for results from injecting ergotamine 2 minutes prior to nursing, suggests a certain level of the drug must be present in general circulation before full inhibition of milk let-down is assured. The other possibility is ergotamine through sympatholytic action and central nervous system depression blocks release of oxytocin from posterior pituitary gland. As neural pathways involved in the milk let-down reflex are obscure, it is perhaps premature to speculate on the site of neural blockage by ergotamine. It has been shown, however, that electrical stimulation of anterior hypothalamus(11) and vagi(12) in lactating sheep and goats, and supraoptico-hypophyseal tract(2,13,14) in lactating rabbits resulted in let-down in milk. It is possible that ergotamine inhibits milk let-down in lactating rats through blockage of one or more of these areas.

Summary. 1. The quantity of milk secured by litters of lactating rats treated with ergotamine 10 minutes before nursing was significantly less than that obtained by control offspring. Oxytocin administered before or after ergotamine restored normal milk let-down. 2. Topical application of ergotamine to living rat mammary tissue resulted in arteriole constriction but did not interfere with normal myoepithelial contraction induced by oxytocin. 3. Results indicate ergotamine does not inhibit milk let-down through vasoconstriction of mammary blood vessels thus prohibiting access of oxytocin to effector myoepithelium. Rather, a competition between ergotamine and oxytocin for the effector tissue or a neural block inhibiting release of oxytocin by posterior pituitary gland seem more probable.

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1. Ely, F., and Petersen, W. E., *J. Dairy Sci.*, 1941, v24, 24.
2. Cross, B. A., *J. Endo.*, 1953, v9, 7.
3. Braude, R., and Mitchell, K. G., *ibid.*, 1952, v8, 238.
4. Whittlestone, W. G., *ibid.*, 1954, v10, 167.
5. Linzell, J. L., *J. Physiol.*, 1953, v123, 32.
6. ———, *ibid.*, 1955, v130, 257.
7. Barger, G., *Ergot and Ergotism*. A Monograph. Gurney and Jackson, London, 1931.
8. Grosvenor, C. E., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 294.
9. Richardson, K. C., *Proc. Roy. Soc. London, sB*, 1949, v136, 30.
10. Linzell, J. L., *J. Anat.*, 1952, v86, 49.
11. Andersson, B., *Acta Physiol. Scand.*, 1951, v23, 8.
12. ———, *ibid.*, 1951, v23, 24.
13. Cross, B. A., and Harris, G. W., *J. Endo.*, 1952, v8, 148.
14. ———, *J. Physiol.*, 1951, v113, 35P.

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Fixation of Carbon Dioxide by *Actinomyces* and *Lactobacillus bifidus*.

(22790)

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In recent studies on fermentation of glucose by *Actinomyces bovis* and *Lactobacillus bifidus*(1) a net fixation of CO_2 was definitely observed in some fermentations and suggested in others. The overall stoichiometry of 2 fermentations by *Actinomyces* indicated that CO_2 was being fixed in a manner consistent with the operation of the Wood-Werkman reaction(2) with the formation of succinic acid. The data suggested that this same reaction was functioning to a minor extent in one strain of *L. bifidus;* but that the major pathway in this organism was one which satisfied the equation: glucose→3 acetic acid. In view of the facts that the same stoichiometry of glucose fermentation occurs in fermentations by *Clostridium thermoaceticum*(3), that carbon dioxide is reduced to acetic acid in the clostridial fermentation(4,5), and that both *Actinomyces* and *Lactobacillus bifidus* have been shown to require carbon dioxide for growth under certain conditions(1), it was decided to conduct fermentations in the presence of carbon dioxide-C^{14} and to determine the extent of incorporation of radioactivity into the carbon atoms of the products.

TABLE I. Anaerobic Fermentation of Glucose plus Carbon Dioxide-C^{14} by *Actinomyces* Strain #10049.*

Substrate or product	μmoles/ml	Cts/min. /μmole
Glucose utilized	34.4	
Carbon dioxide (initial)	33.4	523.4
” ” (final)	14.1	492.8
Formic acid	12.6	50.6
Acetic acid	10.3	.36
–COOH		.23
$-CH_3$		.13
Lactic acid	43.2	n.d.†
Succinic acid	11.0	527.2
–COOH		143.3
$-CH_2-$		120.3
Beta alanine (predicted)		383.9
(found)		377.0

* Fermentation of glucose was done by the fermentation procedure described by Pine and Howell (1) using a 1% glucose casitone medium with starch.

† n.d. = not determined.

Materials and methods. The organisms used were *Actinomyces bovis*, strains ATCC #10048 and #10049, and *Lactobacillus bifidus*, strain #308, obtained from Dr. Paul Gyorgi, University of Pennsylvania Hospital. They were grown on casitone-starch medium with 1% glucose in fermentation tubes as described earlier(1). However, sodium carbonate-C^{14} instead of sodium carbonate-C^{12} was used in the pyrogallol seal to induce anaerobiosis. In aerobic fermentation described in Table II, the culture grew anaerobically 24 hours. The culture was then attached through the top stopper of fermentation tube to a one liter flask containing pure oxygen, and the culture mixed for additional 24 hours with magnetic stirrer. Quantitative determinations of the products were done as described previously(1). Pyruvic acid was identified and estimated by the method of Koepsell and Sharpe(6). Formate was degraded in the presence of acetic acid by oxidation with mercuric sulfate and recovered and counted as barium carbonate. The residual acetic acid was steam distilled and its purity verified by its Duclaux constant. In Table I, acetate was degraded by wet oxidation to determine total specific activity; radioactivity in the carboxyl carbon was determined using the Schmidt degradation(7); and radioactivity of methyl carbon was calculated as the difference. In all other fermentations acetate was recovered as barium acetate and counted as such, applying self absorption corrections determined for barium carbonate. In the fermentation in Table I, succinic acid was separated from lactic acid using a celite column according to procedure of Phares *et al.* (8). Only those fractions were used which gave a negative spot test for lactic acid(9). The succinate was degraded as described below. In all other fermentations, non-volatile acids were chromatogramed on paper (S and S #507) using ammonia-ethanol-water solvent of Cheftel *et al.*(10). Acid bands were demonstrated using brom cresol green (0.05%) adjusted to blue with 0.1 N NaOH. A much more sensitive and satisfactory spray was 0.3% solution of ninhydrin used after the papers were air dried and no longer smelled of ammonia. The color was developed in a 105°C oven for 5 to 10 minutes. This procedure was used in those cases where the acids were not recovered from the paper. The radioactive bands obtained from the *Actinomyces* fermentations were located by radioautograms and were checked for coincidence with the acid bands. The acids were identified by their Rf values and were cut from the paper using the radioautogram as a guide. The single radioactive band obtained from the *L. bifidus* fermentation was located using a hand probe. In all cases there was a wide separation of the lactic acid band from that of the succinic acid. In all cases radioactivity and acid bands were exactly coincident

TABLE II. Fermentations of Glucose plus Carbon Dioxide-C^{14} by *Actinomyces* Strains #10048 and #10049 and by *Lactobacillus bifidus*.*

	Actinomyces 10048 Anaerobic		*Actinomyces* 10049 Anaerobic		*Actinomyces* 10049 Aerobic		*L. bifidus* Anaerobic	
Substrate or product	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM
Glucose utilized	22.8		31.8		31.5		49.5	
CO_2 (initial)		1995.0		1995.0		1730.0		1995.0
(final)		1696.0		1867.0		857.5		1505.0
Formic acid	8.62	119.7	11.7	98.4	15.0	61.8	2.0	1.0
Acetic "	6.52	2.4	8.7	.8	13.3	.6	69.8	.0
Lactic "	24.6	120.5	43.0	72.6	29.9	34.7	49.2	.3
–COOH		118.8		72.2		34.7		—
CH_3CHOH–		1.7		.4		.0		—
Succinic acid	8.18	1169.2	11.2	1301.2	7.04	368.0	.6	529.6
–COOH		159.5		248.4		19.1		20.4
–CH_2-		425.1		402.2		164.9		244.6
Beta alanine (predicted)		1009.7		1052.8		348.9		—
" " (found)		1060.0		1020.0		358.0		—

* Fermentation of glucose was done by fermentation procedure described by Pine and Howell(1) using 1% glucose casitone medium with starch. The procedure for aerobic fermentation is given in text.

with one exception. An apparently non acidic single band of high radioactivity was found running behind and not quite touching the succinate band from the aerobic #10049 fermentation of Table II. The identity of this compound has not as yet been determined. The radioactive compounds were eluted from the paper with water. The samples of succinate so obtained from the *Actinomyces* fermentation were acidified with one or 2 drops of 25% HCl, evaporated to dryness on steam bath, and sublimed *in vacuo*. Melting points were taken and all melted within 2 degrees of an authentic sample of succinic acid and with no depression observed with mixtures of the two. The succinic acid of the *L. bifidus* fermentation was not sublimed but was degraded directly. Lactic acid samples were passed through Dowex 50 resin to convert them to free acid, and were neutralized with $Ba(OH)_2$. Succinic acid was wet ashed using the reagents of Van Slyke *et al.*(11) to determine the average specific activity of its carbon atoms. A sample was then decarboxylated and the specific activity of the carbon dioxide determined according to the procedure of Phares(7) maintaining the temperature at 70°C for one half hour or for one hour at 45°C. Under our conditions, approximately 5 to 7% of the methylene carbons of known succinate-2, 3-C^{14} was obtained as the carboxyl carbon. Attempts to isolate ethylene diamine according to the procedure of Phares and Long(12) failed. Upon the suggestion of Dr. Richard Hendler, National Heart Institute, National Institutes of Health, analyses were made for amino acid in the reaction mixture. An amino acid, presumably beta alanine (13) was found. The reaction mixture was neutralized with 50% NaOH, a few ml of water added, and the Na_2SO_4 was allowed to crystallize out over night at 5°C. Ten ml of ethanol were added, the suspension was centrifuged and the supernatant was removed. The precipitate was washed twice with alcohol, and the alcohol fractions combined. The presence of beta alanine was shown using paper chromatograms run with the acetic acid-butanol-water solvent of Calvin *et al.*(14) and the ethanol-ammonia-water solvent. The former solvent was used to separate the beta alanine from residual succinic acid and trace amounts of ethylene-diamine which were also formed. The beta alanine was eluted with water from such chromatograms; its concentration determined using the ninhydrin procedure of Troll and Cannan(15); and its specific activity was determined by plating and counting directly known amounts of the amino acid. Barium lactate was decarboxylated using chromic acid(16). The residual acetic acid was steam distilled, neutralized with $Ba(OH)_2$ and counted as barium acetate, applying the same correction factors used for barium carbonate.

Results. In initial experiments using *Actinomyces* strain #10049 both the volatile and non-volatile acids were radioactive. A small decrease in the specific activity of the carbon dioxide occurred during the fermentation. Later fermentations using strains #10048 and #10049 and *L. bifidus* showed that each of the 3 fixed from one to 3% of the added counts into the cells and that the volatile acids formed by *Actinomyces* were radioactive while those formed by *L. bifidus* were not. All organisms had incorporated radioactive carbon dioxide into the non-volatile acids but the total counts in this fraction from *L. bifidus* were low. An analysis was then made of a fermentation by *Actinomyces* strain #10049, the results of which are presented in Table I. Again there was observed a small decrease in the specific activity of the carbon dioxide although a net fixation of carbon dioxide occurred. The proportion of formate to acetate to succinate formed was approximately 1/1/1 but the ratio of carbon dioxide fixed to any of these products was approximately 1.54. Relatively low carbon recovery (91%) and a high redox index (1.6) indicated the formation of other products having a high redox value. Attempts to find such products failed and revealed only an additional 0.5 micromole of pyruvate formed per ml. Apparently more carbon dioxide was fixed than was required for the operation of the Wood-Werkman reaction. The specific activity of the succinate was slightly higher than that of the initial carbon dioxide. Upon degradation of the succinic acid, approximately 46% of the total activity was found in the methylene carbons. Isolation of the beta alanine verified these results.

The fermentations were repeated using *Actinomyces* strains #10048 and #10049 and *L. bifidus*. An aerobic fermentation of #10049 was also included as described above. The results are given in Table II. In the 2 anaerobic *Actinomyces* fermentations, the ratio of activity of methylene carbons to carboxyl carbons of the succinic acid was 2.67 and 1.62 for strains #10048 and #10049, respectively. For the aerobic fermentation by *Actinomyces* #10049 and the anaerobic *L. bifidus* fermentation the ratio increased to 8.6 and 12.0, respectively. No significant activity was found in any other products of *L. bifidus*.

In the anaerobic *Actinomyces* fermentations, formate and the lactate carboxyl were of approximately the same specific activity but much lower in specific activity than the final carbon dioxide. The specific activity of the final carbon dioxide in the aerobic fermentations was one half its original activity. Similarly, formate and lactate carboxyl carbon of this fermentation had a specific activity one half that found in the sister anaerobic fermentation. The lactic acid of these fermentations was labeled essentially only in the carboxyl carbon.

In aerobic *Actinomyces* fermentation it appeared that lactate was oxidized to acetate and carbon dioxide with a subsequent decrease in the amount of succinic acid formed. Little fixation of carbon dioxide into the carboxyl carbons of succinic acid occurred in this case. However, the reduction of specific activity in the methylene carbons remained proportional to the decrease in specific activity of the carbon dioxide and again was approximately one half that found in the anaerobic fermentation. In general, the calculated values of specific activity for the beta alanine were approximated by the observed values with the exception of the beta alanine isolated from the succinate of *L. bifidus*. In this case, however, the sample of beta alanine was heavily contaminated with salt and no attempt was made to purify it further.

The results of the fermentation of glucose by *L. bifidus* as shown in Table II may be expressed as being essentially: glucose→lactic acid + 1.41 acetic acid + unknown products. The results obtained with the carbon dioxide-C^{14} showed no incorporation of radioactivity into these products. That the added carbon dioxide was available to and was metabolized by this organism was shown by the high specific activity of the succinic acid which it formed. These results are in agreement with those of Kuhn and Tiedemann(17) who found no significant incorporation of carbon dioxide into lactic and acetic acids. These workers fermented glucose-1-C^{14} with *L. bifidus* and obtained lactic and acetic acids having the same specific activity. They did not degrade these products, however. It would appear therefore that the formation of acetic acid from glucose by this organism involves a molecular rearrangement without the concomitant fixation of carbon dioxide as occurs in *Clostridium thermoaceticum*(4,5) or in *Butyribacterium rettgeri*(18).

It is of interest to note the similarity of labeling of succinic acid found in the *L. bifidus* fermentation and the aerobic fermentation by *Actinomyces* #10049. Similarities of the glucose fermentation by these organisms have been reported previously(1). It is also noted that not only do these fermentations of glucose by *Actinomyces* differ qualitatively from the fermentations of the genus *Lactobacillus* (which do not generally form succinic acid) and from members of the genera *Corynebacterium* and *Propionibacterium* (which form propionic acid) but they also differ from *Propionibacterium* in the mechanism by which they utilize carbon dioxide for succinate synthesis.

It is believed that this is the first report of the net fixation of carbon dioxide with its major incorporation into the methylene carbons of succinic acid. However, the results are similar to those reported by Jefferson *et al.* (19) who found that washed suspensions of *Rhizopus nigricans* fermented glucose in the presence of formate-C^{14} with the formation of succinic and fumaric acids having the greatest radioactivity in the central carbons. However, in this fermentation, lactic acid and ethanol formed had their greatest radioactivity in the methyl carbons while in the presence of carbon dioxide-C^{14} only carboxyl labeled acids were formed. Results similar to those of Jefferson *et al.* were obtained with *Escherichia coli* by Nutting and Carson(20).

Summary. Fermentations of glucose in the presence of carbon dioxide-C^{14} were carried out with strains of *Actinomyces* under aerobic and anaerobic conditions and with *Lactobacillus bifidus*. Fermentation products were isolated and degraded. It was found that succinic acid formed by these organisms had a high percentage of its radioactivity in the methylene carbons. When one strain of *Actinomyces* was grown in the presence of oxygen, 90% of the total activity of the succinic acid was found in the methylene carbons as compared to 62% when it was grown anaerobically.

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1. Pine, L., and Howell, A., Jr., *J. Gen. Microbiol.*, 1956, v15, 428.

2. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., Proc Soc. Exp. Biol. and Med., 1941, v46, 313.

3. Fontaine, F. E., Peterson, W. H., McCoy, E., Johnson, M. J., and Ritter, G. J., *J. Bact.*, 1942, v43, 701.

4. Barker, H. A., and Kamen, M. D., *Proc. Nat. Acad. Sci.*, 1945, v31, 219.

5. Wood, H. G., *J. Biol. Chem.*, 1952, v194, 905.

6. Koepsell, H. J., and Sharpe, E. S., *Arch. Biochem. Biophys.*, 1952, v38, 443.

7. Phares, E. F., *ibid.*, 1951, v33, 173.

8. Phares, E. F., Mosbach, E. H., Denison, F. W., Jr., and Carson, S. F., *Anal. Chem.*, 1952, v24, 660.

9. Feigl, F., *Spot Tests, II Organic Applications*, New York, Elsevier Publishing Co., 1954,

10. Cheftel, R. I., Munier, R., and Macheboeuf, M., *Bull. Soc. Chem. Biol.*, 1953, v35, 1082.

11. Van Slyke, D. D., Plazin, J., Weisiger, J. R., *J Biol. Chem.*, 1951, v191, 299.

12. Phares, E. F., and Long, M. V., *J. Am. Chem. Soc.*, 1955, v77, 2556.

13. Wolf, H. in Adams, R., *Organic Reactions*, 1946, v3, 307.

14. Benson, A. A., Bassham, J. A., Calvin, M, Goodale, T. C., Haas, V. A., and Stepka, W., *J. Am. Chem. Soc.*, 1950, v72, 1710.

15. Troll, W., and Cannan, R. K., *J. Biol. Chem.*, 1953, v200, 803.

16. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E., *Isotopic Carbon*, New York, John Wiley and Sons, Inc., 1949.

17. Kuhn, R., and Tiedemann, H, *Z. Naturforschung*, 1953, v8b, 428.

18. Barker, H. A., Kamen, M. D., and Haas, V. A., *Proc. Nat. Acad. Sci., U.S.*, 1945, v31, 355.

19. Jefferson, W. E., Foster, J. W., Phares, E. F., and Carson, S. E., *J. Am. Chem. Soc.*, 1952, v74, 1477.

20. Nutting, L. A., and Carson, S. F., *J. Bact.*, 1952, v63, 581.

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A Simple Test for Erythropoietin in Deproteinized Plasma Extract.* (22791)

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The effectiveness of a deproteinized plasma extract from rabbits made anemic with phenylhydrazine in producing erythropoietic stimulation has been questioned by several investigators(1,2). Others(3,4,5,6) have confirmed its erythropoietic stimulating properties by a variety of technics. Most recent publications on the subject have shown little rise in Hb or hematocrit in animals receiving the material but have demonstrated statistically significant rises in total red cell volume, red blood count, and nucleated red cells within the marrow. The above technics are cumbersome in that they require long-term injections of recipient animals (2-6 weeks) and require large amounts of extract. Moreover the results, though statistically significant, are not clear cut.

The following method enables a quick test of plasma from an anemic source, requires only small amounts of plasma and enables cross-species examination of the erythropoietic factor in the plasma. Also, utilizing uptake of Fe^{59} into red cells as the criterion for erythropoietic stimulation, the method requires only 3 or 4 days of subcutaneous injections of the deproteinized plasma extract and produces a clear cut differential in Fe^{59} uptake between control and experimental groups of animals. The relative increase of Fe^{59} uptake into the red cell mass of experimental groups as compared with normal controls was considerably greater in hypophysectomized rats than in normal rats.

Materials and methods. a) Young adult Sprague-Dawley rats, aged 2-3 months and ranging in weight from 250-400 g were employed in the experiments utilizing normal animals. The hypophysectomized rats† were of the same age group and had been hypophysectomized for at least 40 days to insure a stable post-hypophysectomized state. They were maintained on a diet consisting of Purina laboratory chow, and bread and milk. b) White New Zealand rabbits were given 1 cc of 1% phenylhydrazine subcutaneously daily until Hb was less than 5 g. They were then bled and the plasma prepared as described previously(5). Control plasma was obtained from normal non-treated rabbits. c) The deproteinized plasma extract was administered in two ways: 1) In unconcentrated form, the extract being diluted to the original volume of plasma. 2) In concentrated form where the extract was diluted to only ⅓ the original volume of plasma. d) The normal rats received 5 cc of deproteinized extract (concentrated or unconcentrated, anemic or normal) for 4 successive days subcutaneously. One hour after the last subcutaneous injection of plasma approximately 1 μc Fe^{59} in 1 cc saline was given intravenously and 24 hr thereafter the animal was bled from the aorta. The radioactivity in the sample was measured in a Nancy Wood well-type scintillation counter. The radioactivity in an aliquot of the original Fe^{59} solution given each animal was similarly measured. Calculation of Fe^{59} uptake into red cells was carried out as previously described(7). Precuation was taken after each experiment to run hematocrits. Any animal with low hematocrit was not included in our results since animals with low hematocrits had tended to run higher iron uptakes. The hypophysectomized rats received 2 cc of plasma extract daily (concentrated or unconcentrated, anemic or normal) for 3 successive days subcutaneously. One hour after the last injection of plasma, 1 μc of Fe^{59} was given intravenously as in the normals and 48 hours thereafter they were bled.

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† Hormone Assay Laboratories, Inc., Chicago, Ill.

TABLE I. Normal Rats Given Deproteinized Plasma Extract Subcutaneously from Anemic and Normal Rabbits for 4 Consecutive Days.

	% uptake of Fe^{59} in RBC, 24 hr after last inj. of plasma				
Weekly groups*	#1	#2	#3	#4	#5
Normal plasma					
Concentrated		35.9	54.2	35.6	66.1
		28.2	56.2	41.4	58.8
		24.3	50.2	56.9	68.0
		32.9	56.3	44.8	71.3
		27.5	49.7	29.5	67.1
		36.1	50.0		47.6
Avg %		30.8	52.7	41.6	63.1
Regular	58.1		57.1	51.5	51.9
	59.8		56.3	43.5	61.4
	39.0		47.7	57.1	56.2
	61.9		49.1	61.3	54.2
	65.6	—	29.8	51.6	52.3
	59.5		53.1	46.7	55.6
	52.1				
	54.8				
	61.4				
	50.5				
	63.8				
	65.7				
Avg %	57.7		48.9	51.9	55.0
Anemic plasma					
Concentrated		47.8	80.6	69.7	67.4
		24.9	91.6	74.1	74.4
		40.0	88.7	78.1	62.2
	—	47.8	88.2	69.7	76.0
		41.8		77.2	64.1
		45.5		70.7	93.1
Avg %		41.3	87.3	73.3	72.9
Regular	69.6		84.4	55.8	64.5
	83.4		71.8	61.8	71.1
	62.5		81.0	40.2	60.8
	69.6		88.5	67.7	50.2
	67.0	—	92.6	60.4	58.0
	74.2		79.6	53.4	53.0
	69.6				
	71.1				
	51.4				
	63.4				
	47.5				
	76.0				
Avg %	67.1		83.0	56.6	59.6

* Weekly groups #1, 2, 3, 4, 5 represent separate experiments performed on five consecutive weeks. Separately prepared extract was administered on each successive week.

Calculation of Fe^{59} uptake into red cells was carried out in the same way as in the normal rats.

Results. The results are shown in Tables I and II. The normal rats showed considerable fluctuation in uptake values from week to week. However, those receiving anemic plasma extract were consistently higher than those receiving normal extract. It should be noted that the groups receiving concentrated anemic extract in all instances had higher average uptake values than the groups receiving unconcentrated anemic material. Also, the concentrated anemic extract was more consistently effective than the unconcentrated material in producing a greater relative response in experimental groups as compared with normal controls. We are unable to explain the wide week to week fluctuation in Fe^{59} uptake in these normal animals.

The hypophysectomized animals showed very distinct differences between the groups receiving normal plasma extract and those re-

TABLE II. Hypophysectomized Rats Receiving Regular and Concentrated Deproteinized Plasma Extract from Normal and Anemic (Phenylhydrazine) Rabbits Subcutaneously for 3 Consecutive Days.

	% uptake of Fe^{59} in RBC, 48 hr after last inj. of plasma		
Weekly groups*	#1	#2	#3
Normal plasma			
Concentrated	.9	1.0	1.5
	1.0	1.2	1.9
	15.4	1.3	9.3
	2.2	4.4	3.0
	7.8	5.2	6.8
Avg %	5.5	2.6	4.5
Regular	3.3	3.6	5.2
	7.8	3.6	1.1
	3.6	6.0	1.3
	1.0	1.7	1.6
		8.8	2.1
Avg %	3.9	4.7	2.3
Anemic plasma			
Concentrated	28.8	26.9	27.6
	45.9	26.8	27.2
	22.0	27.1	27.5
	49.3	21.7	82.0
	26.8	22.9	47.2
		24.6	
Avg %	34.6	25.0	42.3
Regular	18.3	22.7	25.2
	7.5	17.8	21.7
	6.6	14.5	12.6
	12.7	25.8	
	11.7	23.9	
		36.0	
Avg %	11.4	23.5	19.8

* Weekly groups #1, 2, 3 represent separate experiments performed on 3 consecutive weeks. Separately prepared extract was administered on each successive week.

ceiving anemic plasma extract. These differences were considerably more marked than those seen with the normal rats. Groups receiving concentrated anemic plasma had 6 to 9 fold increases in Fe^{59} uptake as compared with those receiving concentrated normal plasma. Likewise, groups receiving unconcentrated plasma had 3 to 8 fold increases as compared with those receiving normal plasma. Again the concentrated material was somewhat more effective than the unconcentrated in stimulating increased Fe^{59} uptake in recipient animals.

Discussion. The above data demonstrate conclusively as judged by Fe^{59} uptake into RBC, the erythropoietic stimulating property of a deproteinized plasma extract from anemic rabbits. Our findings also demonstrate the sensitivity of the hypophysectomized rat as a test preparation for demonstrating the presence of an erythropoietic factor in an extract particularly when incorporation of Fe^{59} into red cells is used as the index of red cell production.

The importance of the deproteinized character of this material is obvious. It enables the testing of erythropoietic activity of plasma from one species into another. More particularly, testing of human plasma from anemia of various sources with regard to its erythropoietic stimulating properties may be possible.

It is of interest that those investigators who have found the deproteinized material inactive have given it intravenously(1,2).[†] Those, on the other hand, who have given it subcutaneously have found it to be effective(3,4, 5,6).

The present results with deproteinized plasma extract from rabbits made anemic with phenylhydrazine demonstrated comparable levels of stimulation to those reported by Freid *et al.*(8) using whole plasma from anemic rats given intravenously to hypophysectomized rats. The criterion of stimulation was the same being the uptake of Fe^{59} in red cell mass.

Summary and conclusions. A simple test for erythropoietin in deproteinized plasma extract is described. A total of 3 or 4 days is required for subcutaneous injection of the plasma extract and only one intravenous injection of Fe^{59} is necessary. When concentrated plasma extracts are given to hypophysectomized rats, 6 to 9 fold differences in response between anemic and control groups are achieved. The deproteinized nature of the material enables cross-species examination of the erythropoietic factor.

1. Erslev, A. J., *ibid.*, 1955, v10, 954.

2. Stohlman, F., Jr., and Mrecher, G., PROC. SOC. EXP. BIOL. AND MED., 1951, v91, 1-4.

3. Borsook, H., Graybiel, A., Keighley, G., and Windsor, E., *Blood,* 1954, v9, 734.

4. Gordon, A. S., Piliero, S. J., Kleinberg, W., and Freedman, H. H., *ibid.*, 1954, v86, 255.

5. Prentice, T. C., and Mirand, E. A., *J. Exp. Med. and Surg.*, 1956, v14, 226.

6. Linman, J. W., and Bethell, F. H., *Blood,* 1956, v11, 310.

7. Elmlinger, P. J., Huff, R. L., and Oda, J. M., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 16.

8. Freid, W., Plzak, L., Jacobsen, L. O., Goldwasser, E., *ibid.*, 1956, v92, 203.

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[†] Recently, in our laboratory, hypophysectomized rats receiving intravenous injections of deproteinized rabbit anemic plasma extract (regular or concentrated) manifested a definite erythropoietic stimulation of a similar magnitude to those receiving this material subcutaneously.

Intracellular Behavior of Brucella Variants in Chick Embryo Cells in Tissue Culture.* (22792)

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Ability of smooth brucellae to localize intracellularly is well known. Brucellae within leucocytes are protected *in vitro* against high concentrations of antibiotics and antiserum for several days(1,2). It has recently been reported, however, that monocytes derived from immune animals restrict intracellular multiplication *in vitro*(3). Non-smooth variants of *Brucella* have not been studied with regard to intracellular residence, although the selective effects of metabolite accumulation and lowered oxygen tension *in vitro* have been thoroughly investigated(4-9). Suppressive effect of selective serum (SS) factor on non-smooth brucellae *in vitro* has also been extensively studied(10). Non-smooth brucellae do not persist long *in vivo* except under conditions where selective metabolites may accumulate, such as in abscessed tissue or aborted fetal material(11-13). Thus, they have been generally considered to be of little significance in brucellosis. However, several instances of mutation from smooth to mucoid *in vivo* have recently been demonstrated, in which the mucoid variants were recovered as often from unabscessed as from abscessed tissue(14).

The present study is concerned with the selective effect of an intracellular environment upon smooth and non-smooth variants of *Brucella*.

Materials and methods. Tissue culture. The culture vessels were 16 x 125 mm screwcap test tubes coated on the inside with a thin film of formvar (polyvinyl formal) according to the method of Barski *et al.*(15). Each culture tube contained 1.5 ml of medium consisting of 20% fresh rabbit serum, 20% chick embryo extract (1/1) and 60% Hanks' balanced salt solution. Ten or 11-day chick embryos (with head, legs and wings removed) were aseptically ground to a fine liquid pulp, and approximately 0.01 ml of this tissue was placed into each culture tube. The tubes were incubated at 37°C in an almost horizontal stationary position. Within 24 hours a heavy growth of fibroblasts covered the lower walls of the tubes. The medium was renewed every 2 or 3 days. *Bacterial cultures.* Brucellae were grown and maintained on *Brucella* agar (Albimi), enteric bacilli on MacConkey agar (Difco) and streptococci on 5% sheep blood agar. The smooth strains of *Brucella* used were *Brucella abortus* a77, a5 (CO_2 dependent) and strain 19 (Lederle no. 1952a); *Brucella suis* s101, s99 and s25, and *Brucella melitensis* m62. I (intermediate), R (rough), M (mucoid) and S^R (smooth-rough) variants of each species were obtained by prolonged growth in *Brucella* broth (Albimi) containing 300 μg/ml DL-α alanine. The acriflavin test(6) was used in conjunction with anti-S antiserum and with colonial observation(4) for differentiation of variants. The streptococcus used was a group A, β hemolytic *Streptococcus pyogenes* freshly isolated following four consecutive passages in mice. The enteric bacilli were laboratory strains of *Escherichia coli, Alcaligenes fecalis* and *Salmonella typhosa*. *Infection of tissue cultures.* The fibroblasts were infected on second day of culture by addition of approximately 10^6 bacteria to the tissue culture medium. Five hours later streptomycin was added to a final concentration of 10 μg/ml to kill extracellular bacteria and to prevent further extracellular growth in the medium. For the streptococcal work 1 unit/ml of penicillin was substituted for streptomycin. For the faster-growing streptococcus and enteric bacilli an infection period of only 2 hours was allowed before addition of antibiotic. At daily intervals after cell infection, a roughly quantitative determination of viable bacteria within the cells was made by scraping a platinum loopful (1/200 ml) of infected tissue from walls of tube and streaking it evenly

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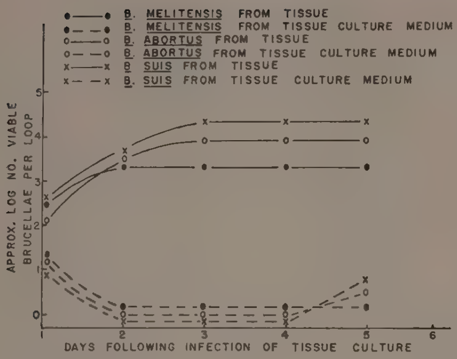

FIG. 1. Three typical experiments showing viable brucellae recovered from infected tissue as compared to those recovered from tissue culture medium in the same tube. The few bacteria appearing in the medium about the fifth day are due to degeneration of some of the infected cells.

on agar plate. A loopful of medium from the same tube was also plated out as a control against presence of extracellular bacteria.

Results. Smooth strains of all 3 species of *Brucella* entered the fibroblasts and not only survived but multiplied therein as shown in Fig. 1. If, however, the tissue cultures were heat-killed (56°C—1 min) either before or after infection, the cells were no longer capable of protecting the brucellae from the streptomycin, and no viable brucellae could be recovered after 30 hours. Thus, under the conditions of the experiment all viable bacteria recovered from tissue cultures represent growth or survival within living avian cells. When formvar-coated cover slips were included in the culture tubes, then withdrawn at intervals and stained by Perrins' modification of the Goodpasteur stain(16), intracytoplasmic bacteria were seen within a small percentage of the fibroblasts. Some fibroblasts contained only a few bacteria after several days infection, while others were nearly filled. To see whether this ability to grow within fibroblasts is unique to brucellae, several other bacterial species were studied under the same conditions. Fig. 2 shows that although all 5 species were phagocytized and thereby survived for a day or two, only the 2 intracellular species *S. typhosa* and *B. suis* multiplied or survived for long.

Prolonged culture of Brucella-infected tissue. Tissue cultures infected with brucellae did not seem to be grossly damaged by this infection. Therefore, prolonged subculture of infected fibroblasts was attempted to determine whether the host cells or the parasite would ultimately prevail over the other. In this experiment the infected fibroblasts were transferred to new tubes every 4 days following trypsinization and washing. Fibroblasts infected with *B. melitensis* m62 and *B. abortus* a5 and a77 were maintained through 7 transfers for 32 days and were still moderately infected when the subcultures were discontinued. Apparently only lightly infected and uninfected fibroblasts survived each trypsinization, since the number of viable brucellae recovered dropped following each transfer, then increased again before the next transfer. Fibroblasts infected with *B. suis* s101 became very granular after 4 to 6 days of infection, became heavily vacuolated and did not survive the second or third trypsinization. The slower growing *B. suis* strains s25 and s99 also eventually destroyed the fibroblasts they infected, although more slowly (after 3 or 4 transfers or 12 to 16 days). Over 10^4 viable brucellae per loop were recovered from cells showing cytopathogenicity due to each of the 3 B. *suis* strains. Prolonged intracellular passage of all 3 species enhanced their colonial smoothness. The colonies recovered developed

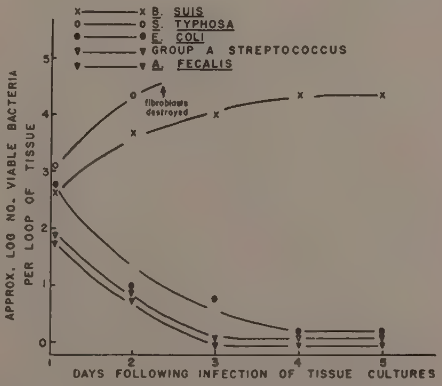

FIG. 2. Comparison of intracellular survival of several bacterial species. *S. typhosa* grew so rapidly intracellularly that cytopathogenicity occurred and streptomycin resistant mutants appeared in about 2 or 3 days.

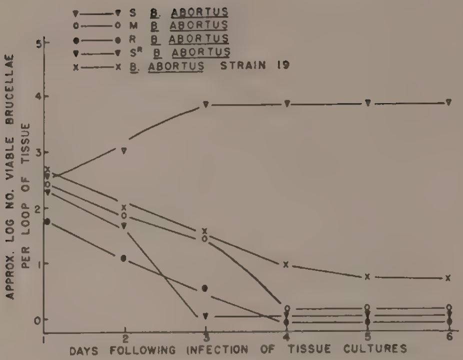

FIG. 3. Intracellular growth of smooth brucellae as compared to non-smooth variants and strain 19 in separate tissue cultures.

more slowly, were less granular and more transparent and bluish.

Intracellular behavior of non-smooth brucellae. M, R, and S^R variants from each of the 3 species were examined for their ability to grow intracellularly as did the smooth parent strains. Strain 19 was also tested. The results of a typical experiment are seen in Fig. 3. It is obvious that neither the non-smooth variants nor strain 19 multiplied or remained viable for long intracellularly. Similar results were obtained with non-smooth variants of *B. melitensis* and *B. suis*. Cells were also infected with mixed inocula of smooth and non-smooth types in various proportions. In all cases the smooth type showed a marked selective advantage (Fig. 4). In another experiment the slimy sediment from a thirty day broth culture of *B. melitensis* was used as an inoculum, and although only non-smooth variants were present on the first 3 days, smooth variants predominated on the fourth day after infection. We next attempted to obtain dissociation from non-smooth to smooth in tissue culture, followed by selection of the smooth type. Therefore, very heavy inocula of non-smooth variants were used to increase the probability of obtaining the S mutant. In some cases this was successful. S colonies were quite often obtained from I inocula and more rarely from M inocula, but never from R or S^R inocula. However, it was observed that very large inocula (over 10^7) of even non-smooth variants caused overwhelming infection, often leading to cytopathogenicity and even to prolonged survival of non-smooth types within the fibroblasts.

Effect of Brucella antiserum and of a alanine in tissue culture medium. It was felt that the presence of the SS factor(10) in the normal rabbit serum of the tissue culture medium might have influenced the results. Therefore, fresh antiserum from rabbits infected with *B. melitensis* was substituted for normal serum. Despite the presence of anti-S antibodies and complement, and despite the absence of the SS factor, the results were not altered. The S type still multiplied intracellularly and the non-S variants were eliminated. Likewise, the addition of DL-a alanine to the tissue culture medium in a final concentration of 300 μg/ml [sufficient to cause rapid selection of non-smooth variants in liquid culture(17)] did not alter the smooth selective character of the intracellular environment.

Discussion. It is apparent that brucellae and other bacteria capable of intracellular growth are in some way resistant to those intracellular mechanisms which inhibit other bacteria such as *E. coli* or the streptococcus. Possibly the loss of surface antigens(18) accompanying mutation from S to non-S might help explain the increased vulnerability of non-smooth brucellae to cellular defenses.

The survival of *Brucella* for long periods

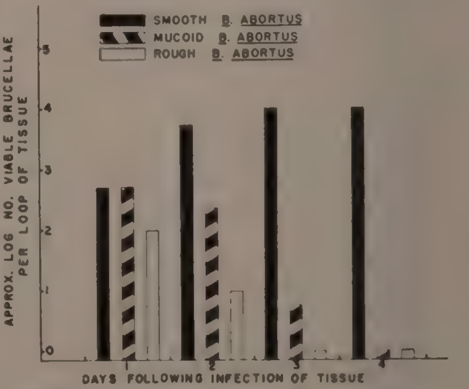

FIG. 4. Intracellular selection of the smooth type in a typical tissue culture infected with a mixed inoculum containing approximately 10^6 each of S, M, and R variants.

within fibroblasts cultured in a bactericidal medium, without gross destruction of the tissue, helps explain the capacity of *Brucella* to cause insidious, chronic disease. This is in contrast to *S. typhosa* which also multiplied intracellularly but which caused rapid cytopathogenicity.

The unaltered smooth selective capacity of fibroblasts cultured in antiserum (lacking in SS factor), indicates one reason why animals lacking the SS factor, and even immune animals, clear non-smooth brucellae from their body much more rapidly than they do the smooth type(14).

It appears that the virulence of *Brucella* is correlated with their ability to multiply intracellularly since the avirulent non-smooth variants and strain 19 are unable to do so. Further work is in progress on the intracellular infection of mammalian cells derived from normal and immune animals.

Summary. 1) Smooth brucellae were ingested by, and multiplied within, fibroblasts in tissue culture. Non-smooth variants and strain 19 neither multiplied nor survived long intracellularly unless the cells were massively infected. Brucellae remained viable intracellularly for over 30 days despite the presence of a bactericidal concentration of streptomycin in the tissue culture medium. Extensive cytopathogenicity was not evident except with *Brucella suis* which caused slow destruction of the fibroblasts. Under the same conditions of culture *Salmonella typhosa* multiplied rapidly intracellularly and caused complete cell destruction within several days. 2) Mixed infections of fibroblasts with smooth and non-smooth brucellae showed the intracellular environment to be highly selective for the smooth type. Mutation from I to S and M to S, followed by selection of the S type, occurred in several heavily inoculated tissue cultures. The addition of anti-S antiserum or DL-α alanine to the tissue culture medium did not affect the smooth-selective behavior of the intracellular environment.

1. Magoffin, R. L., and Spink, W. W., *J. Lab. and Clin. Med.*, 1951, v37, 924.

2. Shaffer, J. M., Kucera, C. J., and Spink, W. W., *J. Exp. Med.*, 1953, v97, 77.

3. Pomales-Lebron, A., and Stinebring, W. H., *Bact. Proc.*, 1956, 93.

4. Henry, B. S., *J. Infect. Dis.*, 1933, v52, 374.

5. Huddleson, I. F., *Am. J. Vet. Res.*, 1946, v7, 5.

6. Braun, W., and Bonestell, A. E., *ibid.*, 1947, v8, 386.

7. Mika, L. A., Braun, W., Ciaccio, E., and Goodlow, R. J., *J. Bacteriol.*, 1954, v68, 562.

8. Sanders, E., and Huddleson, I. F., *Am. J. Vet. Res.*, 1956, v17, 324.

9. Braun, W., Altenbern, R., Kelsh, J., and Sandoval, H., *J. Bacteriol.*, 1956, v71, 417.

10. Cole, L. J., and Braun, W., *J. Immunol.*, 1950, v64, 111.

11. Jones, L. M., and Berman, D. T., *J. Infect. Dis.*, 1951, v89, 214.

12. Braun, W., Gorelick, A., Kraft, M., and Mead, D., *ibid.*, 1951, v89, 286.

13. Simon, E. M., Redfearn, M. S., and Berman, D. T., *ibid.*, 1955, v96, 268.

14. Berman, D. T., Redfearn, M. S., and Simon, E. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 526.

15. Barski, G., Messore, G., and Lepine, P., *Ann. inst. Pasteur*, 1955, v89, 415.

16. Perrin, T. L., *Arch. Path.*, 1943, v36, 559.

17. Goodlow, R. J., Braun, W., and Mika, L. A., *Arch. Biochem.*, 1950, v30, 402.

18. Jones, L. M., *J. Infect. Dis.*, 1953, v92, 26.

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Lack of Hematopoiesis in Tail Bones Transposed to Abdominal Cavity of Hypophysectomized Rats.* (22793)

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Huggins and Blocksom(1) demonstrated that hematopoiesis could be stimulated in the normally inactive terminal tail bones of rats if the ends of the tails were transposed to the abdominal cavity. They had previously(2) shown that the subperiosteal temperature of the terminal vertebrae of the tail was approximately 7 to 12°C lower than the intra-abdominal temperature. They interpreted the establishment of hematopoiesis in the transposed tail bones to be the result of thermal stimulation. Hypophysectomy is known to have a deleterious effect upon peripheral blood and blood-forming organs. Hypophysectomized rats develop a slightly microcytic-hypochromic anemia with little alteration of the total white cell count. The bone marrow of hypophysectomized rats is hypoplastic showing an increase in fat, a decrease in the total number of nucleated cells per mm^3, a reduction in the total number of nucleated erythroid elements per mm^3, and, in the Wistar strain, little effect on white cell precursors (3). It has been shown that the marrow of the hypophysectomized rat is capable of responding to various stimuli similar to the normal rat. An erythropoietic response can be elicited in the marrow of hypophysectomized rats by anoxia, but the anoxic stimulus must be greater than that which will stimulate normal animals(4). The marrow of hypophysectomized rats is also capable of regenerating red blood cells following hemorrhage, but will form only enough to bring the peripheral count back to the pre-bled hypophysectomized levels(5-7). These examples show that even though the marrow is affected by hypophysectomy, a potential to respond to stimuli in a manner similar to normal remains insofar as erythropoiesis is concerned.

The present experiment was conducted to test whether or not hematopoiesis could be initiated in the yellow marrow of the terminal tail vertebrae of hypophysectomized rats, as it is in normal animals, when the tail is transposed to the abdominal cavity.

Material and methods. Adult female rats 3-4 months of age of the Wistar strain were used in the experiment. The animals were fed standard Purina chow supplemented once a week with lettuce. The procedure for transposition of the tails was as follows: A small incision was made in the lateral body wall; a circumferential cut was made in the skin of the tail about 3 inches from the end and the skin of the distal end of the tail was pulled off. The end of the tail was looped forward, inserted into the abdominal cavity through the lateral wall incision, and anchored in place by suturing the tendons of the tail to the muscle wall, and by anastomosing the skin of the tail with the skin of the abdominal wall. Hypophysectomies were performed by the parapharyngeal approach, and the completeness of the operation was determined at autopsy by examination of the organ site under a dissecting microscope. A small sample of blood was removed from the heart, heparinized and studied every 10 days throughout the experiment. Erythrocyte counts were done in duplicate, using U.S. certified blood pipettes and the improved Neubauer counting chamber. Hemoglobin determinations were made in a Klett-Summerson photoelectric colorimeter. Hematocrit determinations were made with Van Allen hematocrit tubes (no diluent) and spun for 1 hour at 2000 r.p.m. Rectal temperatures were taken at 10-day intervals. At autopsy vertebrae from within the abdominal cavity and from the outside loop were removed for study, the outside bone serving as a control along with bones from normal, unoperated rats. The bones were fixed in Helly's fixative, decalcified for 3 days in 5% formic acid, dehydrated for 2 days in graded concentrations of tertiary butyl alcohol, cleared with xylene, embedded in paraffin, cut serially at 6 micra and stained with Giemsa stain buffered to pH 4.75.

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† With technical assistance of Mrs. Louise Brown and Mrs. Nancy Lancaster Martin.

Results. The following groups of animals were studied: (I) normal controls, (II) normal rats with tails transposed for 40, 50, 60 and 70 days, (III) hypophysectomized rats with tails transposed for 70 days, and (IV) normal and hypophysectomized rats without tail transposition kept at a high environmental temperature.

Group I—normal controls—The peripheral blood values and body temperatures for these animals are tabulated in Table I. Section of the tail vertebrae revealed a typical yellow or fatty marrow as shown in Fig. 1. There is a delicate reticulum with occasional isolated hematopoietic cells identifiable in the interstices. The vessels in the marrow may or may not be filled with blood.

Group II—normal rats with transposed tail—The rats of this group can be considered as a unit because the differences in time did not manifest any significant differences in the results. Transposition of the tail had no significant effect on the peripheral blood picture even though red marrow did develop in some of the tail vertebrae (Table I). The body temperature also showed no significant change from the beginning to the end of the experiment (Table I). Vertebrae taken from the outside loop of the tail showed the typical fatty marrow seen in the normal rat (Fig. 1 and 2). The response of the vertebrae from within the abdominal cavity is tabulated in Table II. Thirty-seven of a total of 41 operated animals (90.2%) showed a positive response, a positive response being recorded when there was any accumulation of hematopoietic cells into groups within the interstices of the reticulum. This was sometimes accompanied by an obvious thickening of the reticular framework. A response that was considered "good" is illustrated in Fig. 3. It is characterized by a substantial increase in hematopoietic cells of all types. Responses equal to this were seen at all time intervals

TABLE I. Peripheral Blood Picture and Body Temperatures of Normal Unoperated Control Rats, and Normal and Hypophysectomized Rats with Tails Transposed to Body Cavity.

	Initial values				Final values			
	Erythrocyte count in millions/mm³	Hematocrit in %	Hemoglobin in g/100 cc	Rectal temp., °F	Erythrocyte count in millions/mm³	Hematocrit in %	Hemoglobin in g/100 cc	Rectal temp., °F
Normal controls (6)	8.71 ± .17*	44.7 ± .52	16.1 ± .05	100.5 ± .56	9.39 ± .28	45.0 ± .57	15.9 ± .20	100.4 ± .45
With tail transposed to abdominal cavity† (41)	8.86 ± .06	45.5 ± .33	15.9 ± .10	100.0 ± .20	8.88 ± .08	45.2 ± .35	16.4 ± .10	100.2 ± .22
Hypophysectomized; tail in cavity 70 days (16)	8.74 ± .17	46.3 ± .81	16.6 ± .26	100.4 ± .14	7.53 ± .14	34.8 ± .71	12.0 ± .22	98.6 ± .22

* ± Stand. error.

† Since no significant difference was found among any of the values for the various groups of normal animals with tails transposed, the figures for the 4 groups were combined.

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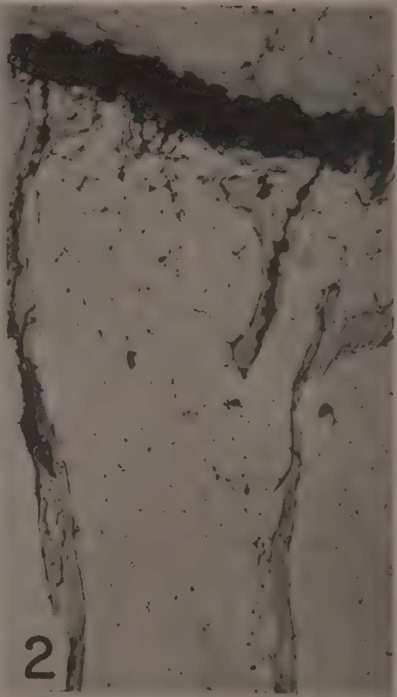
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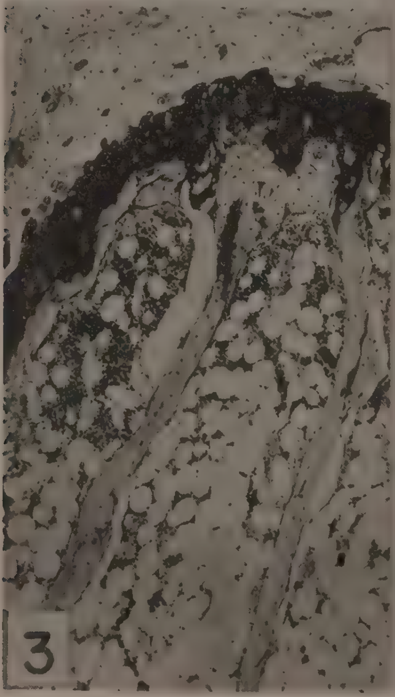
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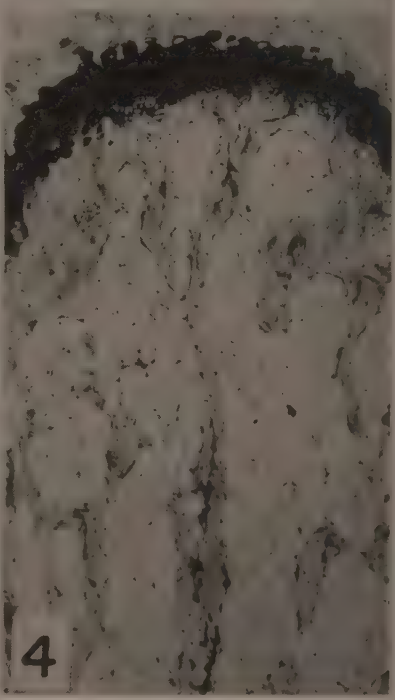
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TABLE II. Hematopoietic Response in Tail Bones Transposed to Abdominal Cavity in Normal and Hypophysectomized Rats.

	No. of rats	No. with			
		Positive response	Erythroid elements	Myeloid elements	Megakaryocytes
Tail in abdominal cavity 40 days	10	10	10	10	9
Idem 50 ”	11	9	9	9	5
” 60 ”	8	7	7	7	4
” 70 ”	12	11	10	11	10
Hypophysectomized; tail in cavity 70 days	16	1	?	1	0

of this operated group. In every case in which there was a response the distribution of the cells was limited to the metaphysis of the bone; increases in vascularity were noted in some but not in all of the vertebrae. Table II shows that the various cell types are well represented in the different groups of operated normal animals. The response was not limited to any one cell type; erythropoiesis, myelopoiesis, as well as megakaryocytic development, was initiated. The observations also agreed with the finding of Huggins and Blocksom in that there was a tendency for a more extensive myeloid distribution than the other cell types.

Group III—hypophysectomized; tail transposed animals—Seventy days after hypophysectomy these animals showed the usual post-hypophysectomy anemia (Table I) and a significant drop (1.8°F) in body temperature. A study of the tail vertebrae from within the body cavity of these animals revealed that only 1 (6.2%) of 16 animals showed any indication of response; the response in this 1 animal was no greater than the minimal response recorded for the control group. No megakaryocytes were seen and identification of erythroid elements was questionable. The remaining animals showed marrows similar to the unoperated control group (Fig. 4).

Group IV—normal and hypophysectomized animals at elevated environmental temperatures—Huggins and Blocksom found that hematopoiesis could be stimulated in the tail bones of normal rats kept at high environmental temperatures. A small experiment was conducted to repeat this work and to extend it to the hypophysectomized animal. Five normal and 5 hypophysectomized rats were kept in an oven at 98°F for a period of 50 days. Only 1 of the 10 rats (a normal animal) showed a response.

Discussion. Huggins and Blocksom postulated that conversion of yellow marrow to red marrow could be accomplished by thermal stimulation, and that red marrow was distributed in those bones having higher environmental temperatures and yellow in those having lower temperatures. The present experiment shows that this effect cannot be shown in the hypophysectomized animal.

Although many changes occur in the hypophysectomized animal which may account for this lack of response, lowered body temperature must be considered as a possible cause. Huggins and Blocksom found that when normal, unoperated animals were placed in an oven for 21 to 70 days, at an environmental temperature of 91 to 96°F, hematopoiesis was stimulated in the tail bones of all of the animals, but the response was not as extensive as it was in the transposed vertebrae. These temperatures are well below the body temperature of the hypophysectomized rat. This suggests, since hematopoiesis can be stimulated at these lower temperatures in the normal animal, that the lowered body temperature in the hypophysectomized rat is not the

FIG. 1. Section from metaphysis of a tail vertebra from a normal rat. ×160.

FIG. 2. Similar section of a tail vertebra taken from the loop of tail lying outside of the body after the end had been transposed to within the abdominal cavity of a normal rat. ×160.

FIG. 3. Similar section of a tail vertebra taken from that portion of tail lying within abdominal cavity of a normal animal for 70 days. ×160.

FIG. 4. Same as Fig. 3 but from a hypophysectomized rat. ×160.

factor limiting stimulation. This same observation, however, was made on only 1 of 5 animals, kept at 98°F, in the present experiment. In addition, it is known that marrow in the hypophysectomized rat is less sensitive to various stimuli, *i.e.*, anoxia(4), and bleeding(5-7); therefore, until such time as hypophysectomized rats are maintained at temperatures equal to normal or even higher than normal, the lowered body temperature cannot be eliminated as possibly having had an influence on the results.

Huggins and Blocksom also showed that anemia induced by repeated bleeding or splenectomy of animals infected with Bartonella did not stimulate hematopoiesis in the tail bones of normal rats, but the accompanying anemia resulted in a more extensive hematopoietic response in bones transposed to the abdominal cavity. The anemia of hypophysectomy does not have this stimulatory effect.

It is of interest to note that in the present experiment hypophysectomy resulted in an inhibition of both erythropoiesis and myelopoiesis. This is in contrast to the results obtained by Meineke and Crafts(3) where, in femur marrow, hypophysectomy induced a depression in erythropoiesis only; there was no effect on myelopoiesis.

It is possible that hypophysectomy has removed some hormone or caused some change in the animal which is necessary for the thermal stimulus to be effective. This could be the direct effect of removal of a single hormone (such as growth hormone), a combination of hormones, or might be the result of a secondary effect on metabolism. Reparative hormonal therapy under conditions outlined in this experiment might be of value in providing information as to the mechanism involved in the initiation of hematopoiesis.

Summary. Thirty-seven of 41 normal female rats, in which the ends of the tails were transposed into the abdominal cavity, showed a stimulation of hematopoiesis in the intra-abdominal vertebrae. Developing cells of all 3 types—erythroid, myeloid and megakaryocytes—were observed in these animals. In similarly treated hypophysectomized female rats only 1 of 16 animals showed any indication of response. The experiment provides another example of the depressed hematopoietic potential of the hypophysectomized rat.

1. Huggins, C., and Blocksom, B. H., Jr., *J. Exp. Med.*, 1936, v64, 253.

2. Huggins, C., Blocksom, B. H., Jr., and Noonan, W. J., *Am. J. Physiol.*, 1936, v115, 395.

3. Meineke, H. A., and Crafts, R. C., *Anat. Rec.*, 1956, v124, 47.

4. Feigin, W. M., and Gordon, A. S., *Endocrinology*, 1950, v47, 364.

5. Querido, A., and Overbeek, G. A., *Arch. Internat. Pharmacodyn. et de Therp.*, 1939, v61, 475.

6. Finkelstein, G., Gordon, A. S., and Charipper, H. A., *Endocrinology*, 1944, v35, 267.

7. Silbergleit, A., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 170.

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Resistance to Bacteria in Hemorrhagic Shock. VII. Demonstration of Leucotoxin in Plasma of Shocked Rabbit.* (22794)

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In the first of this series of studies it was shown that the phagocytic index of granulocytes from the peritoneal cavity of the normal dog, immersed in plasma from the dog in hemorrhagic shock, falls more and more the longer the dog has been in shock when the

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test plasma is taken(1). This was attributed to a progressive decline in the potency of phagocytosis-promoting factors in the plasma. But it would have been just as valid to interpret the data as signifying a progressive rise in the potency of a phagocytosis-inhibiting factor in the plasma. In the present communication we report evidence that the plasma of the shocked rabbit contains a substance that inflicts functional and morphologic damage upon normal phagocytes *in vitro.*

Method. The unchallenged peritoneal cavity of a normal or shocked rabbit, when washed with iced gelatin Locke's solution, yields a cell suspension containing 70% macrophages. Thus one can obtain and compare these cells from the normal and shocked rabbit. But a comparison of granulocytes is not possible, because they appear in large numbers and as the predominant cell after several hours in response to an irritant injected into the peritoneal cavity of the normal rabbit, but they do not appear in significant numbers in response to the same stimulus in the shocked rabbit, either during shock or for several hours after an effective transfusion. The reason for the failure of granulocytes to leave the circulation in the shocked rabbit may be because a severe granulocytopenia develops soon after shock is induced and persists for some hours, even after an effective transfusion. For the same reason the blood is an unpromising source of a sufficient supply of these cells. We have, therefore, obtained data on the effect of plasma from normal and shocked rabbits upon the morphology and function of (a), macrophages from the peritoneal cavity of the normal and shocked rabbit and of (b), granulocytes from the peritoneal cavity of the normal, but not of the shocked rabbit. By a technic described elsewhere(2) macrophages were obtained from the peritoneal cavities of normal and shocked rabbits, and granulocytes were obtained from the peritoneal cavities of normal rabbits. They were promptly harvested by centrifugation for 20 minutes at 4°C, washed 3 times in iced gelatin Locke's (g-L) solution, and resuspended in a final concentration of 10 million cells per 0.1 ml of the g-L solution. Plasma was obtained from normal rabbits and from rabbits which were either in advanced shock (irreversible to transfusion), or in shock of 2 hours duration (reversible shock). In the latter it was taken at the end of the 2 hours, just before transfusion, or 4 hours after the transfusion. The g-L solution and the plasma were heparinized (4 mg/100 ml). *For study of comparative morphology of cells in normal and shock plasma,* 0.5 ml of plasma was added to 0.1 ml of cell suspension and the mixture incubated for one hour at 37°C. Smears of the mixture were then made and stained with methylene blue (for nuclei) or Wright's stain (for cytoplasm). All preparations were made in duplicate. Granulocytes were classified as mildly injured when the nucleus showed a change from the multilobular to an oval or crescent shape and a shift to the periphery of the cytoplasm. More severe injury consisted of distortion of the cytoplasm, with blurring of detail and loss of granularity, and conversion of the nucleus to a crescent shaped amorphous mass displaced to the periphery of the cytoplasm. Such cells resemble L. E. cells. The percent of injured cells was determined from counts of 200 cells in each preparation. The mildly injured cells were counted separately from the severely injured ones. Less than 2% of granulocytes freshly garnered from the peritoneal cavity and suspended in the g-L solution show mild or severe injury. After they are allowed to incubate in the g-L solution or in normal plasma for one hour at 37°C, not more than 5% show such injury. *To determine reversibility of morphologic injury* to granulocytes, the shock plasma and cell mixture was incubated at 37°C for one hour, then washed 3 x in iced g-L solution, and then resuspended in the g-L solution or in normal plasma, and again incubated for one hour at 37°C. Smears were made after the first and second incubation, and any change in the percentage of injured cells noted. *For study of comparative phagocytic and bacteriostatic power of phagocytes* in normal and shocked plasma the following technic, adapted from Wood and Smith(3), was employed: 0.1 ml of cell suspension was added to 0.01 ml of a 5 hour culture of Friedlander's bacillus (about one million bacteria). To this mixture 0.1 ml of normal or shock plasma was added. (Since storage for some days at –20°C was found not to change the effect of plasma on the behaviour of phagocytes, the test plasma was either fresh or stored.) Aliquots (0.015 ml) of this mixture were incubated on 1 cm squares of filter paper in a moist Petri dish at 37°C for one hour. One square was then smeared on a glass slide, and the latter stained with methylene blue. The percentage of bacteria ingested, and the percentage of cells with ingested bacteria were determined by counts of the intracellular and extracellular bacteria in fields containing a total of 200 cells. The remaining filter paper preparations were pooled and washed until clean with 0.5 to 1.0 ml of iced g-L solution. The washings were transferred to a centrifuge tube and spun for 5 minutes at 800 rpm in a refrigerated centrifuge to precipitate the cells. The supernatant was decanted and spun for 15 minutes at 2500 rpm to harvest the uningested bacteria. The cell precipitate was triply washed in iced g-L solution to remove uningested adherent bacteria. The washed cell precipitate, to which 1 ml of iced g-L solution was added, was then transferred to a mortar containing sterile sand, and ground for 15 seconds in order to rupture the cells and release the ingested bacteria. The mixture was transferred with added iced g-L solution to a centrifuge tube, and spun for 5 minutes at 800 rpm to remove the sand and cellular debris. The ingested bacteria were now free in the supernatant, which was decanted and spun at 4°C for 15 minutes at 2500 rpm to harvest these bacteria. Separate "agar smears" were made from the precipitates of uningested and of ingested bacteria. These smears were prepared by spreading a minute sample of each precipitate on a coverslip previously coated with a layer of melted agar, then covering the smear with another layer of melted agar. The coverslips were then transferred to a covered Petri dish and incubated for 2 hours at 37°C. They were then stained with methylene blue, inverted, mounted on a glass slide and sealed with paraffin. Counts were made of the number of single bacteria (dead[†]) and of colonies (live) and the percent of dead bacteria determined.

TABLE I. % of Granulocytes Showing Morphologic Injury after Incubation for One Hour at 37°C in Shock Plasma[*] in 6 Experiments.

Reversible shock plasma, no transfusion			Reversible shock plasma, 4-6 hr after transfusion			Irreversible shock plasma, no transfusion		
M[†]	S[‡]	T[§]	M	S	T	M	S	T
20	50	70	15	15	30	27	57	84
16	14	30	40	15	55	20	14	34
18	15	33	20	10	30	18	14	32
23	34	57	30	30	60	18	18	36
			30	25	55	31	37	68
			20	60	80			

[*] Effect of normal plasma was determined in every case simultaneously with effect of shock plasma on the same cell suspension. Because normal plasma did not produce damage to more than 5% of the cells in any case, data for normal plasma are omitted.

[†] M—Mild injury. [‡] S—Severe injury ("L.E." cells). [§] T = M + S.

Some 6 determinations of the phagocytic and of the bacteriostatic activity were made in duplicate in each of the following categories: 1, normal macrophages in normal plasma; 2, normal macrophages in shock plasma; 3, macrophages from shocked rabbits in normal plasma; 4, macrophages from shocked rabbits in shock plasma; 5, granulocytes from normal rabbits in normal plasma; and 6, granulocytes from normal rabbits in shock plasma.

Results. A. *Effect of shock plasma on morphology of phagocytes.* (Fig. 1 and 2). 1. *Macrophages.* These cells appear normal when freshly harvested from the unchallenged peritoneal cavity of the shocked as well as of the normal rabbit. No change from normal was observed after standing for one hour at 37°C in either g-L solution or in normal plasma. But after standing at 37°C for one hour in plasma from rabbits in advanced shock some 20% showed damage consisting of any or all of the following changes: Small and large vacuoles in the cytoplasm, loss of definition of the cell wall, pyknosis of the nucleus, or blurring of its membrane. 2. *Granulocytes* (Table I). Not more than 1-2% of these cells show abnormal morphology when examined immediately after harvesting from the peritoneal cavity. After these cells are immersed in g-L solution or normal plasma for one hour at 37°C, the percentage of damaged cells does not exceed 5%. Table I lists the data on plasma from reversibly shocked and irreversibly shocked rabbits. Even if one

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[†] Single bacteria might be inhibited rather than dead. But the general significance of the observation is valid, whichever is the case.

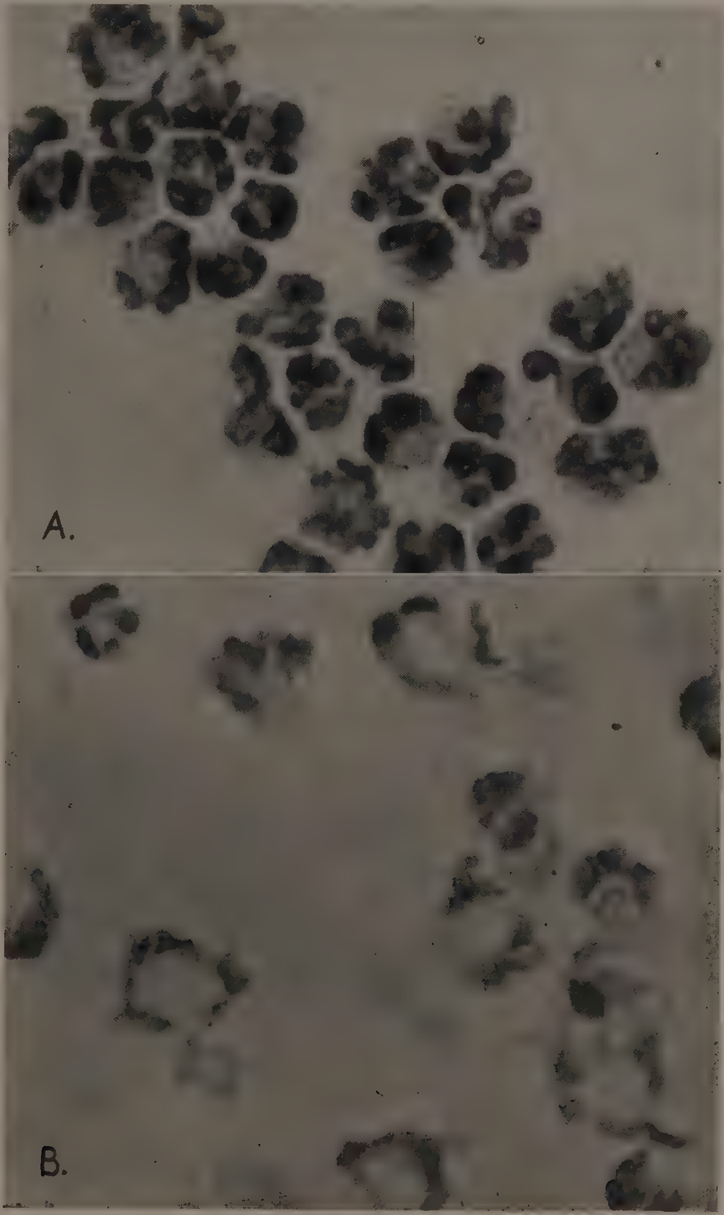

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FIG. 1. A. Granulocytes from peritoneal cavity of normal rabbit washed in iced gelatin-Locke's solution, and then suspended in fresh plasma from a normal rabbit. B. Same as A, except that plasma is from a rabbit in hemorrhagic shock of 2 hr duration.

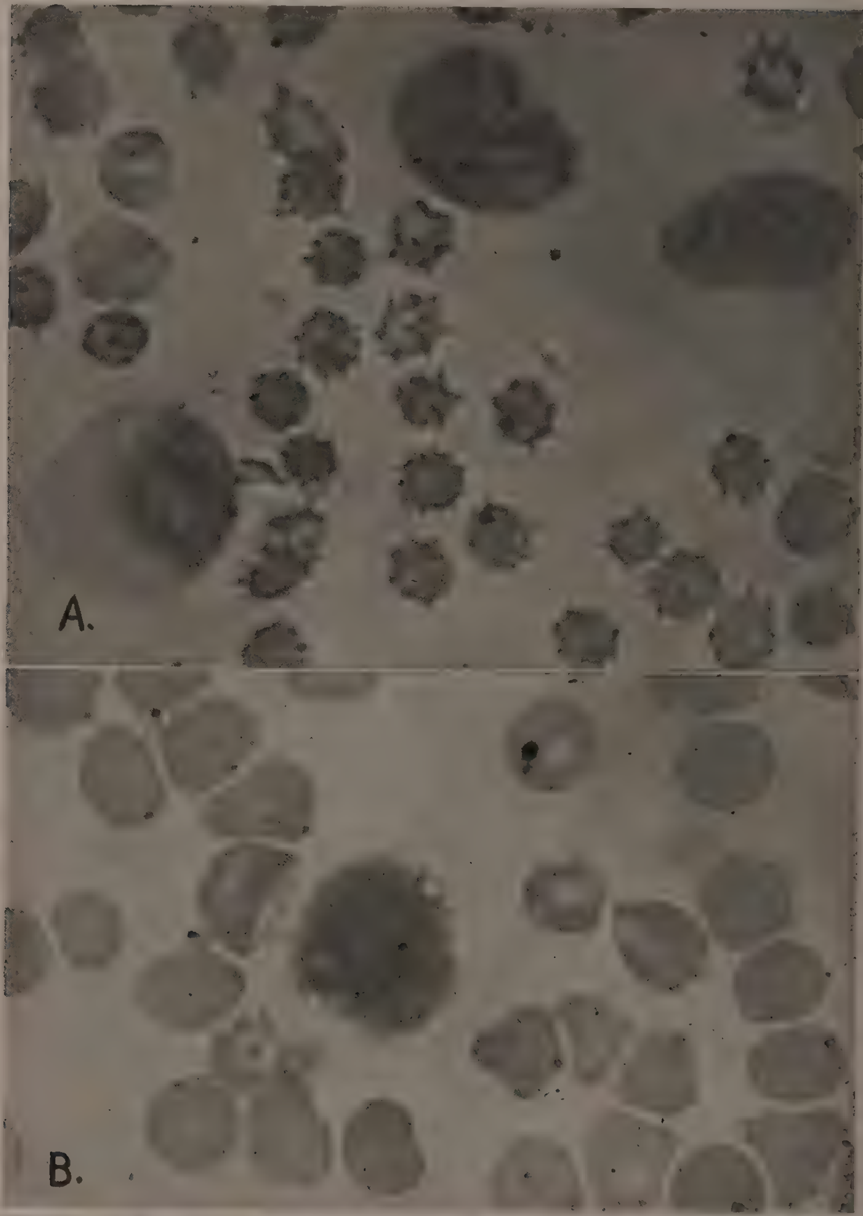

FIG. 2. A. Macrophages from peritoneal cavity of normal rabbit—washed in iced gelatin—Locke's solution and then suspended in fresh plasma from normal rabbit. B. Same as A except that plasma is from a rabbit in hemorrhagic shock of 2 hr duration.

TABLE II. % of Normal Granulocytes Showing Injury after Incubation for One Hour at 37°C in Reversible Shock Plasma followed by Incubation for One Hour in g-L Solution or Normal Plasma. 6 experiments.

Reversible shock plasma, 4-6 hr after transfusion			Gelatin, Locke's sol.			Normal plasma		
M*	S†	T‡	M	S	T	M	S	T
15	15	30	5	3	8	5	0	5
40	15	55	6	5	11	5	3	8
20	10	30	2	2	4	3	3	6
30	30	60	5	13	18	11	2	13
30	25	55	5	5	10	8	4	10
20	60	80	8	18	26	5	15	20

* M—Mild injury.
† S—Severe injury ("L.E." cells).
‡ T—M + S.

TABLE III. Comparison of Differential Leucocyte Count in Circulating Blood after 2 Hr of Hemorrhagic Shock with Differential Leucocyte Count in Blood in Elevated Reservoir.

Exp. No.	Type of cell	In circulating blood before shock	In circulating blood after 2 hr of shock	In elevated reservoir
1	P*	67	41	73
	L†	27	55	23
	M‡	6	4	4
2	P	67	31	68
	L	30	69	29
	M	3	0	3
3	P	61	19	45
	L	38	79	54
	M	1	2	1
4	P	51	34	60
	L	47	66	39
	M	2	0	1

* P—Polymorphonuclear leucocytes.
† L—Lymphocytes.
‡ M—Mononuclear cells.

is inclined to accept the "L.E." cells only as sufficiently significant evidence of morphologic injury, there is no doubt that shock plasma is distinguishable from normal plasma by the presence in shock plasma of a cell damaging factor.

Reversibility of morphologic injury. That the morphologic damage is reversible is shown in Table II, which compares the percentage of cells damaged by irreversible shock plasma with the percentage of the same cells washed free of this plasma and subsequently exposed for one hour at 37°C to g-L solution or to normal plasma. The technic of the shock experiment, as we perform it(4), allows continuous exchange between the blood in the circulation of the animal and the blood lost by the animal into an elevated reservoir. It is conceivable that the blood, which stands in the reservoir at room temperature for several hours, may generate a toxic factor that is factitious, and that such a toxic factor, rather than one developing in the animal, accounts for the effect of shock plasma upon the integrity of the phagocytes. That this is not so is indicated by the data in Tables III and IV, which show (1), that the granulocytopenia which develops in shock does not affect the differential count of the blood in the reservoir (Table III); (2), that the number of granulocytes in the latter remains near normal (Table III); (3), that the percent of normal granulocytes damaged by the animal's plasma after 2 hours of shock is substantially greater than the percent damaged by the plasma in the reservoir (Table IVA); and (4) that the phagocytic and bacteriostatic potency of normal granulocytes immersed in plasma from the reservoir after 2 hours of shock is distinctly greater than that of the

TABLE IV-A. Comparison of % of Mildly and Severely Damaged Granulocytes Immersed in Plasma from Circulating Blood and in Plasma from Blood in Elevated Reservoir after 2 Hr of Hemorrhagic Shock. 4 experiments.

Plasma from circulating blood			Plasma from blood in reservoir		
M*	S†	T‡	M	S	T
18	15	33	20	4	24
23	34	57	6	7	13
20	50	70	3	4	7
16	14	30	10	3	13

* M—Mild injury.
† S—Severe injury.
‡ T—M + S.

TABLE IV-B. Comparison of Phagocytic and Bacteriostatic Potency of Normal Granulocytes Immersed in Plasma from Blood in Reservoir after 2 Hr and in Plasma from Circulating Blood after 2 Hr in Shock. 4 experiments.

Phagocytic index		Bacteriostatic index	
SP*	RP†	SP	RP
10	45	30	49
18	27	15	44
14	50	19	50
23	40	32	76

* SP—Reversible shock plasma.
† RP—Plasma from blood in reservoir.

TABLE V. Effect of Irreversible Shock Plasma on Percentage of Bacteria Ingested by Phagocytes.*

Normal macrophages† in			Shock macrophages‡ in			Normal granulocytes§ in		
N.P.‖	S.P.¶	$\frac{\text{S.P.}}{\text{N.P.}}$**	N.P.	S.P.	$\frac{\text{S.P.}}{\text{N.P.}}$	N.P.	S.P.	$\frac{\text{S.P.}}{\text{N.P.}}$
35	18	.51	34	20	.60	53	26	.49
44	31	.70	37	30	.80	42	21	.50
33	20	.60	37	16	.43	50	32	.64
31	16	.51	30	17	.56	31	19	.61
55	34	.61	50	21	.42	60	27	.45
—	—	.59	—	—	.56	—	—	.54

* Phagocytic activity = % of bacteria in field of 200 intracellular phagocytes. Figures are in %.

† Macrophages harvested from unchallenged peritoneal cavity of normal rabbit immediately after killing.

‡ Macrophages harvested from unchallenged peritoneal cavity of rabbit killed 4 hr after transfusion for shock of 2 hr duration.

§ Granulocytes from peritoneal cavity of normal rabbit killed 6 hr after inj. of beef infusion broth.

‖ N.P.—Normal plasma. ¶ S.P.—Plasma from rabbit shortly before death from hemorrhagic shock, not responsive to transfusion.

$$** \quad \frac{\text{S.P.}}{\text{N.P.}} = \frac{\text{Phagocytic index in shock plasma}}{\text{Phagocytic index in normal plasma}}.$$

same cells immersed in the plasma from the circulating blood taken at the same time.

B. *Effect of shock plasma on phagocytosis. Macrophages.* From Table V one can conclude that: (1) there is no significant difference between the percent of bacteria ingested by macrophages taken from the normal rabbit and the percent of bacteria ingested by macrophages from the rabbit dying of irreversible shock. (2) The percent of bacteria ingested by macrophages from the normal and shocked rabbit and by normal granulocytes is depressed to about the same extent by shock plasma, *i.e.* by somewhat less than 50%. (3) The deficient phagocytosis is due to a toxic factor in the plasma of the shocked animal. Table VI shows that the shock plasma reduces the percentage of cells with ingested bacteria. We have no data to show whether the reduced phagocytic index is due to the total suppression of phagocytosis by some cells or to a reduction in the average number of bacteria ingested per cell. To determine whether the amount of inhibiting factor depends upon the duration of shock, we have compared the degree of depression of the phagocytic indexes in the various types of shock plasma in Table VII. No significant difference between the toxicity of the plasma of reversible and irreversible shock appears in the data except those on the activity of granulocytes, which, though suggestive, are too meager to permit a definitive inference.

TABLE VII. Comparative Depression of Phagocytic Activity of Phagocytes by Plasma from Rabbits in Reversible and Irreversible Shock.*

	Normal macrophages in		Shock macrophages in		Normal granulocytes in	
	R′SP†	ISP‡	R′SP	ISP	RSP§	ISP
	.51	.56	.60	.50	.66	.49
	.70	.70	.80	.63	.74	.50
	.60	.41	.43	.46	.70	.64
	.51	.71	.56	.60	.62	.61
	.61	.59	.42	.65	—	.45
Avg	.60	.60	.56	.57	.68	.54

* Figures in each case represent ratio of phagocytic index of cells in shock plasma to phagocytic index in normal plasma.

† R′SP—Plasma taken 4 hr after transfusion for 2 hr of shock.

‡ ISP—Plasma taken from rabbits before death from advanced shock. No transfusion.

§ RSP—Plasma taken after 2 hr of shock. No transfusion.

C. *Effect of shock plasma on bacteriostatic potency of phagocytes.* Table VIII lists the percentage of ingested bacteria rendered bacteriostatic by phagocytes after immersion for one hour at 37°C in normal plasma, and in plasma from reversibly and irreversibly shocked rabbits. The depression of the bacteriostatic potency of these cells by the various shock plasmas is expressed in terms of the

TABLE VI. Effect of Shock Plasma on Percentage of Cells Containing Bacteria.

Normal macrophages in			Normal macrophages in			Shock macrophages in			Shock macrophages in			Normal granulocytes in			Normal granulocytes in		
		R'SP			ISP			R'SP			ISP			RSP			ISP
NP*	R'SP‡	NP	NP	ISP§	NP	NP	R'SP	NP	NP	ISP	NP	NP	RSP†	NP	NP	ISP	NP
40	38	.95	22	16	.73	52	20	.40	20	16	.80	30	18	.60	41	20	.49
30	19	.63	23	16	.69	28	20	.71	23	14	.61	40	30	.75	30	18	.60
33	25	.75	41	33	.80	34	18	.71	40	18	.45	33	31	.94	39	31	.80
25	25	1.00	30	27	.90	20	18	.53	28	25	.89	24	18	.75	20	18	.90
33	20	.60	24	18	.75	18	20	.90	27	18	.66	—	—	—	41	29	.70
34	25	.74	—	—	—	30	25	1.11	—	—	—	—	—	—	—	—	—
Avg		.78			.77			.73			.68			.76			.70

* NP—Normal plasma. † RSP—Plasma taken after 2 hr of shock. No transfusion. ‡ R'SP—Plasma taken 4 hr after transfusion for 2 hr of shock. § ISP—Plasma taken from rabbits before death from advanced shock. No transfusion.

TABLE VIII. Effect of Shock Plasma on Bacteriostatic Potency of Phagocytes.

Normal macrophages in						Shock macrophages in						Normal granulocytes in								
		R'SP			ISP			R'SP			ISP			RSP			R'SP			ISP
NP*	R'SP†	NP	NP	ISP‡	NP	NP	R'SP	NP	NP	ISP	NP	NP	RSP§	NP	NP	R'SP	NP	NP	ISP	NP
49	29	.59	37	20	.54	50	30	.66	40	18	.45	68	30	.44	60	28	.47	55	27	.49
40	30	.75	—	—	—	46	25	.61	41	25	.61	51	15	.30	67	31	.46	71	30	.42
51	25	.49	50	27	.54	50	22	.44	37	20	.54	46	19	.41	57	29	.57	64	24	.37
35	25	.71	40	29	.73	30	25	.83	22	15	.68	80	32	.40	48	20	.42	51	18	.35
38	29	1.76	25	15	.60	40	32	.80	40	20	.50	—	—	—	—	—	—	68	31	.46
24	20	.59	38	21	.55	28	20	.63	—	—	—	—	—	—	—	—	—	—	—	—
Avg		.65			.59			.65			.56			.39			.47			.51

* NP—Normal plasma. † R'SP—Plasma taken 4 hr after transfusion for 2 hr of shock. ‡ ISP—Plasma taken from rabbits before death from advanced shock. No transfusion. § RSP—Plasma taken after 2 hr of shock. No transfusion.

ratio of the percentage of ingested bacteria that did not multiply when exposed to shock plasma to the percentage of ingested bacteria that did not multiply when exposed to normal plasma. These data demonstrate a considerable degree of damage to the bacteriostatic potency of phagocytes by shock plasma. They also demonstrate that the injury is about the same whether the shock is reversible or irreversible, and that the toxin in the plasma is still as active 4 hours after transfusion for 2 hours of shock as it is immediately after 2 hours of shock. Furthermore, it appears that the leucotoxin reduces the bacteriostatic potency of granulocytes to a greater degree than of macrophages.

Discussion. From the foregoing data we conclude that a leucotoxin is present in the plasma of hemorrhagic shock. This conclusion rests chiefly upon the evidence relating to the functional behaviour of the phagocytes. Since we have no data on the functional capacity of the morphologically damaged phagocytes, we cannot say whether the morphologic injury bears any relation to the functional injury. Nor can we say that the leucotoxin responsible for the functional injury is the substance causing the morphologic injury.

What is the source of the leucotoxin? The evidence given is sufficient to exclude a substance that might be produced in consequence of the special conditions of our shock experiment. The phagocytes and the plasma in the relatively stagnant blood in the reservoir have been found to be comparable in behaviour to the phagocytes and the plasma from the blood of the normal rabbit. And the transfusion has been excluded as a factor in the toxicity of the plasma of the animal recovering from shock, and of the animal dying of shock after a transfusion.

Is the leucotoxin an endotoxin? Endotoxins given intravenously are known to produce granulocytopenia. We have data to be published which show that endotoxins, added to a mixture of normal plasma and normal granulocytes in concentrations equivalent to those in circulating blood after injecting a sublethal dose, produce morphologic damage which is virtually the same as that produced by plasma from the shocked animal. Endotoxins also damage the antibacterial defense mechanisms and produce typical irreversible peripheral vascular collapse(5). Elsewhere(6) we have put forward the view that hemorrhagic shock weakens the antibacterial defense, in consequence of which unfettered bacterial activity eventually inflicts irreversible damage to the peripheral vessels and prevents them from responding to transfusion. Weakness of the antibacterial defense can presumably result from tissue hypoxia alone. But the presence of a leucotoxin in the blood must add to the damage to the antibacterial defense. Thus the way is open for the production of endotoxins from invading bacteria, whether the leucotoxin itself is or is not an endotoxin. Consequently there is reason to assume that a vicious circle is set up, and that toxin begets more toxin, with resulting injury to the peripheral vessels which is indistinguishable from that known to be caused by the direct injection of endotoxins into a healthy animal(5).

According to this thesis the toxicity of the plasma of the shocked rabbit just before death should be greater than that of the plasma of the rabbit which is still reversible to transfusion. We have already referred to evidence that this is so in the dog, but we do not find it so in the rabbit. This may be because our assay technic is too crude, or because there is in fact no necessary relationship between the amount of circulating toxin and the development of irreversibility to transfusion in the rabbit. For in this species we have found that the effect of a given amount of endotoxin on the peripheral vascular system is related not only to the dose of endotoxin, but to the sensitivity to the endotoxin, which increases enormously as the shock continues (7).

Summary and conclusions. 1) Normal macrophages from the peritoneal cavity of the normal and shocked rabbit, and granulocytes from the irritated peritoneal cavity of the normal rabbit show no significant morphologic injury after immersion in normal rabbit plasma for one hour at 37°C. But after immersion in plasma from rabbits in hemorrhagic shock, a considerable percent of the cells show morphologic injury. A large number of granulocytes show changes similar to those characteristic of the L.E. cells. These changes are reversible if the shock plasma is replaced by normal plasma. 2) Shock plasma produces a severe depression of the phagocytic index; the percent of bacteria ingested is some 40% less than in normal plasma, and the percent of cells containing bacteria is some 30% less than in normal plasma. Shock plasma also reduces the bacteriostatic power of the phagocytes. The granulocyte in this respect appears to be damaged more severely than the macrophage. The extent of the injury produced by the plasma from rabbits in advanced shock appears to be no greater than that produced by the plasma of rabbits after two hours of shock, *i.e.* while they are still responsive to transfusion. The injurious property of plasma of the reversibly shocked rabbit persists, with undiminished potency, for at least four hours after transfusion. 3) It is concluded that a leucotoxin develops in the blood of the rabbit in hemorrhagic shock, and that this leucotoxin severely impairs the antibacterial potential of the animal.

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1. Schweinburg, F. B., Yashar, J., Aprahamian, A., Davidoff, D., and Fine, J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 587.

2. Rutenburg, S. H., and Fine, J., *ibid.*, 1956, v91, 217.

3. Smith, M. R., and Wood, W. B., Jr., *J. Exp. Med.*, 1947, v86, 257.

4. Fine, J., *The Bacterial Factor in Traumatic Shock*, pp43-46, C. C. Thomas, Springfield, Ohio, 1954.

5. Thomas, Lewis, *Physiological Disturbances Produced by Endotoxin. Ann. Rev. Physiol.*, 1954, v16, 467.

6. Fine, J., *N. Y. Acad. Sci.*, in press.

7. Schweinburg, F. B., and Fine. J., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 589.

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Reduction of Androstane-3,17-dione by Liver Homogenates.[*] (22795)

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Androstane-3, 17-dione is formed *in vitro* from androstane-3α, 17β-diol by homogenates of the liver and kidney of the rat, guinea pig and rabbit(1), from 4-androstene-3, 17-dione by guinea pig liver and kidney homogenates (2) and from androsterone and epiandrosterone by guinea pig liver homogenates(3). Furthermore, androstane-3, 17-dione has been postulated as the precursor of urinary androsterone and epiandrosterone(4). It would appear that androstane-3, 17-dione occupies a key position in the intermediary metabolism of androgens. Therefore, the metabolism of this substance by liver homogenates has been investigated.

Methods and procedures. Purification of androstane-3, 17-dione.[†] The material was submitted to adsorption chromatography on alumina (washed Harshaw). Those fractions which showed only androstane-3, 17-dione on paper chromatography (0.5 mg/spot) were pooled, treated with Darco charcoal, and recrystallized from acetone and water until a constant melting point was obtained. The purity of the material used in most of the experiments was tested further by infra-red analysis.[‡] *DPNH and FDP.*[§] The DPNH

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[†] The steroids were generously provided by Ciba Pharmaceutical Products, Inc., and Syntex, S. A.

[‡] Analyses were performed with a Perkin-Elmer, Model 21, Infra-red Spectrophotometer.

[§] The following abbreviations are used: DPN, diphosphopyridinenucleotide (Pabst Laboratories); DPNH, reduced DPN; FDP, fructose diphosphate (Nutritional Biochemicals Company); NA, nicotinamide (General Biochemicals, Inc.).

was prepared from DPN by the method of Green and Dewan(5) with some alterations. Reductive conversions of 90% were obtained only when DPN:dithionite ratios of 2:1 were employed. The barium salt of FDP was converted to the sodium salt(6). Livers were obtained from adult male guinea pigs, 6 to 10 months old, which were killed by a blow at the base of the skull and bled. The procedure for incubation and isolation of steroid materials was as previously described(7) except that the separation of ketonic from non-ketonic materials with Girard's T reagent was omitted. The fractions obtained after chromatography on alumina were pooled in accordance with their elution properties and a 0.5 mg aliquot of the pooled fractions was submitted to qualitative analysis by paper chromatography(8). Fractions consisting of mixtures or containing non-steroidal impurities were submitted to paper chromatography for separation and purification. In the case of the principal metabolites, purification was carried out until satisfactory melting points and infra-red spectra were obtained. In addition, derivatives were prepared. The fractions were assayed by a modified Zimmerman reaction(9).

Results. Metabolites. Androsterone and epiandrosterone were the principal conversion products. Androstan-17β-ol-3-one and androstane-3α, 17β-diol were detected in much smaller amounts by paper chromatography in some of the experiments. The following is an example of the physical properties of the metabolites. The values obtained for the authentic samples are given in parentheses. Androsterone, m. 186-7° (185-6°); mixed, m. 186-8°; acetate, m. 165-7° (166-8°); oxime, m. 213-15° (214-17°). Epiandrosterone, m. 167-73° (174-5°); mixed, m. 165-72°; oxime, m. 183-4° (185-7°); mixed, m. 184-7°. The infra-red spectra were identical with those of the authentic compounds.

Homogenate. The amounts of metabolites formed were never very large, ranging from 0.4 to 1.2% in the case of androsterone, and from 0.8 to 2.5% in the case of epiandrosterone (Table I). No significant variation in the amounts of metabolites formed was observed when the pH was varied from 5.2 to 8.0, or when the length of the incubation period was varied to 2.5 hours. These results indicated that either only a small amount of apoenzyme for the reduction of the 3 and none for the 17 ketone group was present or that the amount of endogenous co-factor was not adequate to provide a substantial reduction. It is known that under similar conditions oxidation of the 17-hydroxyl of testosterone is greatly accelerated by the addition of DPN(10) with an optimum at about 4 mg/g of guinea pig liver (unpublished). The endogenous DPN of guinea pig liver, however, is only about 0.5 mg/g and the DPNH even lower, 0.1-0.2 mg/g(11,12). Therefore, it seemed reasonable to assume that the homogenate (apoenzyme) needed exogenous DPNH to become fully effective.

TABLE I. Reduction of Androstane-3,17-dione by Guinea Pig Liver Homogenates.*

	Exp. No.	Metabolites† And., %	Epi., %	Recovered androstane-dione, %
Boiled controls	3	0	0	86.2 ± 1.3
Experimental	4	.9 ± .2	1.8 ± .4	84.2 ± 2.4

* Adult non-fasted male guinea pigs used. Steroid (100 mg), 12 g liver and 50 ml Krebs-Ringer bicarbonate buffer, pH 7.3 (one exp. at 7.5) mixed in Waring blendor for 2 min. Mixture transferred quantitatively by washing with 50 ml buffer to Fernbach flask. Incubation at 27° for 2.5 hr; 95% O_2–5% CO_2 was the gaseous phase. Values are means and their stand. errors.

† And. = Androsterone; Epi. = Epiandrosterone.

Effect of co-factors. Since DPNH can be formed effectively by liver homogenates from FDP and DPN(6), this procedure was utilized (Table II). DPN alone did not increase the yield of metabolites. The addition of FDP with DPN, however, produced a substantial increase in the production of epiandrosterone but not androsterone. A further increase in the formation of epiandrosterone and also an increase in androsterone was secured by increasing the pH from 5.1 to 6.7. The same results were observed when an atmosphere of nitrogen was used. The omission of nicotinamide, however, prevented the increased production of both metabolites presumably due to a destruction of the nucleo-

TABLE II. Influence of pH, Fructose Diphosphate (FDP), Diphosphopyridine Nucleotide (DPN) and Gaseous Atmosphere in Reduction of Androstane-3,17-dione by Guinea Pig Liver Homogenates.

Exp. No.	FDP, mg*	pH	Metabolites§ And., %	Epi., %	Aa., %	Total recovery, %‡
			Air atmosphere			
1		5.2	1.0	1.8		89.7
1	170	5.2	2.2	5.8		86.6
2	330	5.1	1.1	4.8		91.9
1	170†	6.5	2.1	1.7		96.6
2	250	6.7	6.0	9.0	.4	83.6
			N. atmosphere			
2	250	6.7	5.8	8.9	.3	95.2

* Liver (12 g) from adult non-fasted male guinea pigs and 50 ml 0.2 M Na_2HPO_4 buffer mixed in Waring blendor for 20 sec. Mixture transferred with 50 ml of buffer to Fernbach flask containing 50 mg steroid, 50 mg DPN and 400 mg NA. Incubation at 37° for 1.5 hr. Air was the gaseous phase.

† NA omitted.

‡ Metabolites plus recovered androstanedione. Control in which liver was boiled gave a recovery of 86.1%.

§ And. = Androsterone; Epi. = Epiandrosterone; Aa. = Androstanolone.

tides by the endogenous nucleotidase(13,14).

Effect of pH. Since pH seemed to be a factor in the yield of metabolites, studies were carried out to determine the optimum pH of the reductase when DPNH was used. The maximum formation of epiandrosterone occurred at about pH 6.7 (Fig. 1), and the yield was approximately twice that observed when FDP and DPN were used (Table II) but the amount of androsterone not only was less than that obtained at pH 6.7 with FDP and DPN but also was not affected by the change in pH. Small but significant amounts of androstan-17β-ol-3-one were formed.

Amount of DPNH. The addition of more than 19 mg of DPNH to the nicotinamide fortified liver homogenate at pH 6.6-6.7 produced only a slight further increase in the formation of epiandrosterone and had no further effect on the yield of androsterone (Fig. 2).

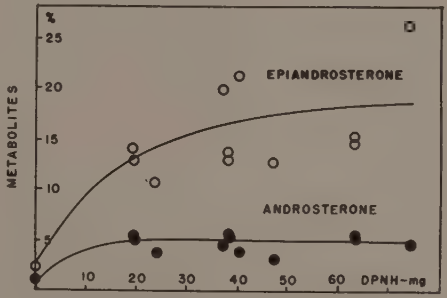

FIG. 2. Effect of quantity of DPNH on reduction of androstane-3,17-dione by guinea pig liver homogenate at pH 6.6-6.7 in presence of 400 mg NA. Incubation was 1.5 hr at 37° in air atmosphere. Total recoveries were from 91-98%. A boiled control yielded no metabolites and 86.1% recovery of androstanedione.

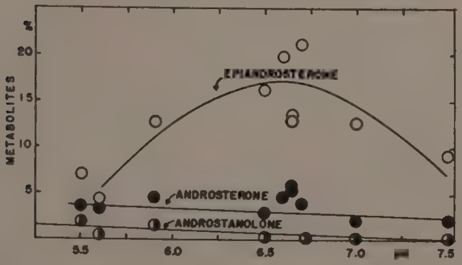

FIG. 1. Effect of pH on reduction of 50 mg of androstane-3,17-dione by 12 g guinea pig liver homogenate in presence of from 37 to 40 mg DPNH and 400 mg NA. Incubation for 1.5 hr at 37° in air atmosphere.

Distribution of androstane-3, 17-dione reductive activity. The liver homogenate was centrifuged at 1500 x g for 5 minutes at 3-8°.‖ The pellet fraction was resuspended in buffer for use. Approximately 75% of the ability to reduce androstane-3, 17-dione remained in the supernate (Table III). The re-

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‖ Centrifugations were performed on an International Refrigerated Centrifuge, Model PR-2 provided by the American Cancer Society.

TABLE III. Distribution of Androstanedione Reductive Activity in Adult Male Guinea Pig Liver.

Fraction	Metabolites recovered*			Recovered	Total re-
	And., %	Epi., %	Aa., %	Ae.,* %	covery, %
Homogenate	4.6 ± .0	23.0 ± 3.2	trace	57.0 ± .0	84.6 ± 3.2
Supernatant	3.6 ± .8	15.5 ± .1	"	70.5 ± 3.1	89.6 ± 4.0
Sediment	2.7 ± .3	4.7 ± 2.5		88.4 ± .0	95.8 ± 2.8

* And. = Androsterone; Epi. = Epiandrosterone; Aa. = Androstanolone; Ae. = Androstanedione.

Tissue (12 g) and 50 ml 0.2 Na_2HPO_4 buffer (pH 6.6) mixed for 20 sec. in Waring blendor. Centrifugation 5 min. at 3-8° and 1500 × g; supernatants were decanted and sediments resuspended in 50 ml buffer. DPNH added in 50 ml solution. Each flask contained 50 mg steroid and 400 mg NA. Incubation was for 1.5 hr at 37°. Air was the gaseous phase. Results are avg of 2 series of experiments in which 37 and 74 mg of DPNH were used.

mainder of the activity was present in the resuspended pellet. There appeared to be a greater sedimentation of the androsterone-forming activity than of the epiandrosterone-forming activity.

Discussion. The reduction of androstanedione to androsterone and epiandrosterone apparently is accomplished by two different enzyme systems. A similar separation has been demonstrated in preparations of Pseudomonas(15).

Summary. Guinea pig liver homogenates reduce androstane-3, 17-dione to androsterone, epiandrosterone, androstan-17β-ol-3-one and androstane-3α, 17β-diol. The latter two compounds were observed only in trace amounts when addition of co-factors to the incubation mixture produced a marked increase in the amount of metabolites formed. Anaerobic conditions had no effect on the conversion produced by DPN, FDP and Na. The system which produced epiandrosterone exhibited an optimum pH of about 6.7. The formation of the other metabolites, however, was not affected by changes in pH. Approximately three-fourths of the ability to reduce androstane-3, 17-dione was in the supernatant fraction of homogenates centrifuged at 1500 x g.

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1. Kochakian, C. D., and Aposhian, H. V., *Arch. Biochem. Biophys.*, 1952, v37, 442.

2. Kochakian, C. D., and Stidworthy, G. H., *J. Biol. Chem.*, 1954, v210, 933.

3. Carroll, B. R., Hamm, D. I., and Kochakian, C. D., *Proc. Am. Assn. Cancer Research*, 1955, v2, 8.

4. Dobriner, K., *Acta De L'Union Internationale Contre Le Cancer*, 1948, v6, 315.

5. Green, D. E., and Dewan, J. G., *Biochem. J.*, 1937, v31, 1069.

6. Wald, G., and Hubbard, R., *J. Gen. Physiol*, 1949, v32, 367.

7. Clark, L. C., Jr., Kochakian, C. D., and Lobotsky, J., *J. Biol. Chem.*, 1947, v171, 493.

8. Kochakian, C. D., and Stidworthy, G. H., *ibid.*, 1952, v199, 607.

9. Holtorff, A. F., and Koch, F. C., *ibid.*, 1940, v135, 377.

10. Sweat, M. L., Samuels, L. T., and Lumry, R. J., *ibid.*, 1950, v185, 75.

11. Strength, D. R., Ringler, I., and Nelson, W. L., *Arch. Biochem. Biophys.*, 1954, v48, 107.

12. Gabriel, O., Schwarz, O. F., and Hoffman-Ostenhof, O., *Monatsh*, 1954, v85, 840.

13. Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, 1947, v35, 502.

14. Handler, P., and Klein, J. R., *J. Biol. Chem.*, 1942, v143, 49.

15. Talalay, P., and Dobson, M. M., *ibid.*, 1954, v205, 1197.

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Neuraminic Acid as a Constituent of Human Cerebrospinal Fluid.* (22796)

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Recent investigations on the distribution of neuraminic acid (NA) have revealed that its occurrence in the body is not restricted to its role as a constituent of water-soluble glycolipids of brain(1,2), nor to states of disturbed glycolipid metabolism(1,6), but that NA occurs in a wide variety of body organs and fluids without necessarily constituting an integral unit of a glycolipid molecule(2,3,4,8). Thus, Böhm *et al.*(7,8) have isolated NA from serum, and have shown that normally it is tightly bound to serum proteins, being wholly precipitable with trichloracetic acid. These findings were confirmed by Uzman and Rosen(9) who also established that the bulk of serum NA is bound to alpha-2 globulins, and present in non-dialyzable form though not as a constituent of gangliosides. The NA values for normal serum recorded by Böhm *et al.*(7) was 40-65 mg/100 ml serum. Those recorded by other workers(9) ranged between 44 and 49.6 mg per 100 ml serum. Thus, assuming a normal serum protein of 6g%, the NA would be expected to correspond to approximately 0.67-1.1% of the total serum protein according to Böhm *et al.*(7) and 0.78-0.83% according to Uzman and Rosen(9). If the NA in the cerebrospinal fluid (CSF) were solely derived from serum, the amount of CSF neuraminic acid would be expected to be approximately proportional to the protein content of the CSF, provided that specific protein fractions having a high NA content were not preferentially concentrated in the CSF. It is a well known fact, however, that the bulk of CSF protein consists of albumin, with the globulin fractions constituting a much smaller proportion of the total than in serum(10-12). Evidence for enrichment of specific glycoproteins with high NA content, like the fetuin (6% NA) of Klenk(13) and the mucoprotein (8% NA) of Winzler(13, 14), in human CSF is totally lacking. The extremely low values (2.4 ± 0.9 μg NA per ml CSF) reported(15) on the basis of analysis of CSF total glycoproteins is further evidence against any selective increase of NA-rich glycoproteins in CSF with respect to serum.

The present investigation was undertaken to establish whether a relationship between the NA and protein content of human CSF did indeed exist, and to ascertain if, in addition, the CSF contained NA in a different state of binding than that found in serum. The latter consideration assumes importance in view of the possibility of release of NA from tissue glycolipids in the course of cerebral metabolism. Our findings demonstrate that human CSF contains 3 to 20 times more NA than would be expected from the CSF protein content, and that only the non-dialyzable NA moiety of the total NA reflects that portion of CSF neuraminic acid derived from serum proteins.

Material. The samples studied represented cerebrospinal fluid obtained by spinal puncture in the course of pneumoencephalography(10-30 ml), myelography (ca. 10 ml) or routine spinal fluid examinations, as well as samples obtained during diagnostic ventriculography (10-15 ml) from unselected cases studied on the Harvard Neurological Unit at the Boston City Hospital. All samples grossly contaminated with blood, or containing more than 5 white cells/cu mm were rejected.

Methods. The determination of neuraminic acid was effected by the method outlined by Böhm *et al.*(7). Total neuraminic acid was usually determined on 3 ml samples of CSF. 3 ml of Bial's reagent was used. This modification did not affect the linearity, if a 3 ml water blank was used, and blank recordings subtracted from the test samples. Duplicates

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checked within ± 1.0%. Non-dialyzable NA was determined by subjecting 3 ml of CSF to dialysis in 27/32 inch viscose tubing overnight. The dialyzed CSF was quantitatively recovered from the dialysis bags (with distilled water washings), made up to a known volume and aliquots taken for NA determination. These were compared with the total NA, and expressed as per cent non-dialyzable NA. Duplicates agreed within ± 2.0%. TCA-precipitable NA was determined by adding 3 ml of 10% TCA to 3 ml of CSF, centrifuging off the ensuing precipitate, and determining the NA content in the precipitate in the manner originally described by Böhm *et al.*(7).

The CSF total protein was determined by the sulfosalicylic acid method of Ayer *et al.* (16) as modified by Merritt and Fremont-Smith(17), inasmuch as this procedure gave the most reliable and reproducible results in the protein concentration ranges involved (duplicates agreed within 1%). The possibility of incomplete precipitation of some glycoproteins is a handicap shared by all other methods of CSF protein determination. In those cases where the partition of neuraminic acid was studied in the serum as well as in the CSF of a subject, the alpha-2 globulins were also determined by paper electrophoresis, using the Spinco-Durrum electrophoresis apparatus, quantitation being effected by use of the Spinco "Analytrol" photometric recording scanner and integrator.

Results. In 49 samples of human CSF examined, the neuraminic acid concentration was found to vary between 10.7 and 33.5 μg per ml. In those samples containing normal amounts of protein (15-30 mg%) the neuraminic acid corresponded in amount to about 10% of the protein (on the average). The concentration of NA did increase with increasing amounts of total protein in the CSF, but there was no proportional relationship. On the other hand, the NA/Total Protein ratio decreased with increasing protein concentration, so that in samples with a total protein above 500 mg%, the value of NA as per cent of total protein approached 1.0%, which is close to the average value in serum (0.8%).

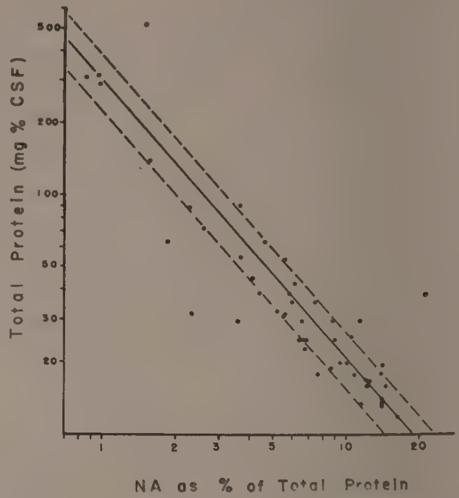

FIG. 1. Showing variation of NA-protein relationship in CSF with increasing conc. of CSF protein.

Thus, although some of the CSF neuraminic acid content is dependent on the NA accounted for by serum proteins, this constitutes a minor portion. Fig. 1 represents the relationship of the CSF neuraminic acid expressed as per cent of total protein to the CSF total protein concentration. It is evident that NA as % total protein decreases with increased protein concentration and rises with decreased protein concentration. Furthermore, this relationship is an exponential one both with the ordinate as well as the abscissa of the graph, so that the majority of values fall along a straight line when plotted against logarithmic ordinate and abscissa. The 5 exceptions represent gross deviations, presumably depending on the pathology presented by the patients involved. In CSF samples with very low protein content (6-10 mg%) obtained by ventricular drainage the NA was proportionately much greater, so that it represented 20-30% of the total protein (top 3 points on Fig. 2).

In those samples where the non-dialyzable NA was estimated, the results indicated that it is this fraction which is derived from serum. In Fig. 2, the log of the non-dialyzable NA, as per cent of the total NA, is plotted against the log of total neuraminic acid as per cent of the total protein. It is evident that as the

TABLE I.

Serum alpha-2 globulins as % total protein	CSF total protein, mg %	NA as % of total protein		Non-dialyzable NA as % total NA		TCA-precipitated NA as % total NA	
		Serum	CSF	Serum	CSF	Serum	CSF
12.8	62	.82	4.6		40.0	95.0	
11.4	13	.81	14.4		21.0	92.4	
12.6	43	.63	6.12	96.5	31.0	88.0	17.85
11.2	64	.49	1.84	100.0	43.0	88.5	22.9
12.1	56	.63	3.66	100.0	43.0	88.0	
9.8	20	.84	9.35	99.5	23.8	92.0	11.7

non-dialyzable NA fraction increases and approaches 100%, the NA/protein ratio drops to levels approaching those prevalent in serum since the NA value expressed as per cent of total protein approaches the serum value of 1.0%. Similarly, low values for per cent non-dialyzable NA correspond to those samples that had low total protein and therefore a high NA/protein ratio.

It is also evident that the relationship of non-dialyzable neuraminic acid to the NA/protein ratio is an exponential one, inasmuch as the values (excepting those marked with arrows), plotted in Fig. 2 fall along a straight line if logarithmic ordinate and abscissa are used. The 4 values, indicated by arrows, that deviate grossly from the straight-line trend represent an extreme type of pathological deviation, the significance of which remains to be elucidated.

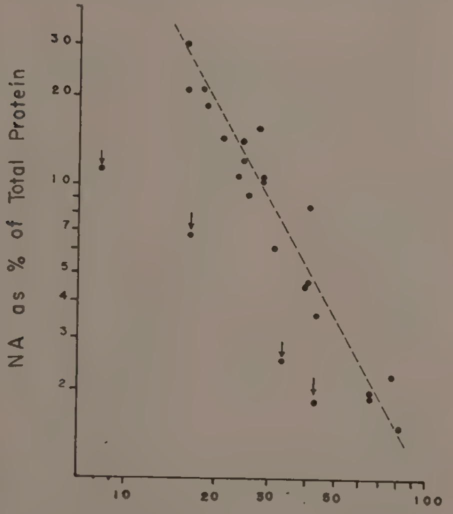

FIG. 2. Variation of non-dialyzable NA moiety of CSF with changes in total NA/protein ratio. Dotted line represents theoretical curve that would be obtained if the whole non-dialyzable NA fraction of the CSF were derived from serum.

That the variations in the NA/protein ratio and the per cent of non-dialyzable NA fraction in the CSF are not due to variations of these values also in the serum of any individual subject is demonstrated by the comparison of values for serum and CSF in 6 randomly selected subjects (Table I). It is apparent that the different state of binding and different NA/protein ratio in the CSF cannot be explained by the NA/protein ratio of the serum. Nor was there any significant difference in the serum alpha-2 globulin concentrations to account for the elevated NA/protein ratios in the CSF. Similarly, the different proportions of the nondialyzable NA fraction in the CSF in contrast to the serum could not be explained on the basis of differences in the alpha-2 globulin content of the sera of the same subjects.

Discussion. From the foregoing information it appears that human cerebrospinal fluid is notable for the high proportion of dialyzable neuraminic acid it contains with respect to its total NA content. The disparity between the dialyzable and bound neuraminic acid in CSF on one hand, and serum on the other, most probably is explained by considerations involving a 2-fold origin for the CSF neuraminic acid. One of these, accounting for the non-dialyzable neuraminic acid fraction, is probably derived from the serum neuraminic acid carried into the CSF by serum proteins. The relationship of the non-dialyzable fraction to the CSF total neuraminic acid with increasing protein levels (NA as per cent of total protein) as indicated in Fig. 2, clearly supports this view. The second source of neuraminic acid could be the metabolic processes of the central nervous system itself which, presumably in the course of ganglioside turnover, may contribute to the dialyzable, and probably free, neuraminic acid moiety. The fact that it is the dialyzable fraction that is responsible for the disproportionate elevation of NA with respect to protein in CSF clearly argues against the high NA/protein ratio of CSF as being due to selective entry of specific glycoproteins with high NA content. Admittedly, none of the samples studied can be assumed to represent entirely normal values for human CSF, inasmuch as there is always suspicion, if not evidence, of neurological disease when a spinal or ventricular puncture is performed. This fact had, however, the advantage of providing a survey of variations of neuraminic acid content with different levels of CSF protein, and establishing the relationship of the non-dialyzable fraction to the serum-derived neuraminic acid, as shown by our data in Figs. 1 and 2. A further advantage would be evinced in the correlation of neurological pathophysiology and gross deviations from the straight-line trend depicted in Fig. 1. Any increase in NA concentration due solely to increase in CSF total protein would be predicatable within the margin of variation indicated by the dotted lines[†] in Fig. 1 by the relationship

$$\log (TP) = -1.16 \log (NA) + 2.4771$$

which is the equation for the solid line, where (TP) represents total protein in mg% CSF, and (NA) stands for NA as per cent of total protein. Gross deviations from this numerical relationship, plotted in the same manner as in Fig. 1, would indicate a decreased amount of dialyzable NA if the points fall to the left of the line, and an increased amount of dialyzable NA, if the points fall markedly to the right of the line. It is hoped that extensive correlative studies between the gross deviations of the neuraminic acid concentration from those values expected from the total protein content and the neurological conditions giving rise to these states will prove fruitful in defining the source for the free (or dialyzable) moiety of neuraminic acid in the CSF.

Conclusions. 1. The neuraminic acid (NA) content of 49 samples of human cerebrospinal fluid (CSF) was studied. In all instances the quantitative relationship of neuraminic acid to the cerebrospinal fluid protein was far in excess of that prevalent in serum. In 26 samples in which the total protein was 30 mg % or less, the neuraminic acid was 4-18 times in excess of that expected if it were derived from serum and bound to protein, as is the case in blood. Unlike its state in serum, most of the CSF neuraminic acid is in freely dialyzable form (60-80%). That moiety of CSF neuraminic acid which is derived from serum is in protein-bound, non-dialyzable form. 2. The origin of the dialyzable NA is unknown, but is not related to ingress of serum proteins into the CSF. The implications of these findings are discussed.

[†] Arbitrarily chosen to include 80% of values. The normal spread is obviously a function of two variables, *i.e.* CSF protein concentration and dialyzable NA, and can only be determined by a study of a very much larger case population.

1. Klenk, E., *Biochemistry of the Developing Nervous System,* Ed. H. Waelsch, Acad. Press, N. Y. (1955), pp. 397-410.

2. Klenk, E., Faillard, H., Weygand, F., and Schöne, H. N., *Z. physiol. Chem.,* 1956, v304, 34.

3. Zilliken, F., Braun, G. A., and György, P., *Arch. Biochem. Biophysics,* 1955, v54, 564.

4. Klenk, E., and Lauenstein, K., *Z. physiol. Chem.,* 1952, v291, 147.

5. Klenk, E., Faillard, H., and Lempfrid, H., *ibid.,* 1955, v301, 235.

6. Uzman, L. L., *A.M.A. Arch. Path.,* 1955, v60, 308.

7. Böhm, P., Dauber, S., and Baumeister, L., *Klin. Wochschr.,* 1954, v32, 289.

8. Böhm, P., and Baumeister, L., *Z. physiol. Chem.,* 1955, v300, 153.

9. Uzman, L. L., and Rosen, H., *Science,* 1954, v120, 1031.

10. Kabat, E. A., Moore, D. H., and Landow, H., *J. Clin. Invest.,* 1942, v21, 571.

11. Baudouin, A., Lewin, J., and Hillion, P., *Compt. rend. soc. Biol.,* 1953, v147, 1036.

12. Ewerbeck, H., *Klin. Wochschr.,* 1950, v28, 692.

13. Klenk, E., and Stoffel, W., *Z. physiol. Chem.,*

1955, v302, 286.

14. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.*, 1950, v185, 561.

15. Roboz, E., Murphy, J. B., Hess, W. C., and Forster, F. M., PROC. SOC. EXP. BIOL. AND MED., 1955, v89, 691.

16. Ayer, J. B., Dailey, M. E., and Fremont-Smith, F., *Arch. Neurol. Psych.*, 1931, v26, 1038.

17. Merritt, H. H., and Fremont-Smith, F., *The Cerebrospinal Fluid*, W. B. Saunders Co., Philadelphia, 1938, pp. 256-260.

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Effect of Dietary Fat on Requirement of Vitamin B_{12} by the Chick.* (22797)

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The young chick's dietary requirement for vit. B_{12} may be influenced by several factors. That the hen's diet affects the vit. B_{12} requirement of her progeny was recognized even before isolation of the vitamin. The requirement of chicks from hens depleted of their vit. B_{12} stores has been shown by most workers to fall between 15 and 30 γ/kg diet(1-4). The vit. B_{12} requirement of chicks from hens fed typical breeder diets was determined by Davis and Briggs(5) to be 1.5 to 2 γ/kg of diet. Similarly, Miller, Norris, and Heuser (6) found that chicks with moderate stores of vit. B_{12} upon hatching required 1.25 γ/kg diet. These values are somewhat below 8 γ, the level that has been tentatively recommended by the Committee on Animal Nutrition of the National Research Council(7). The relationship between vit. B_{12} intake of the hen and requirement of the chick has been quantitated by Milligan, Arscott, and Combs (8). They found that chicks fed a basal corn-soybean oil meal diet (25% protein) require 27, 12, 3, and 0 γ vit. B_{12}/kg diet when the dam's diet contained 0, 4, 8, and 16 γ per kg, respectively.

The influence that certain nutrients of the chick's diet may have upon the requirement of vit. B_{12} has been reviewed by Smith(9). Spivey, Briggs, and Ortiz(10) reported that a severe vit. B_{12} deficiency could be produced in undepleted chicks by the addition of 20% fat to a corn-soybean oil meal diet. The present paper deals with the increase in the vit. B_{12} requirement of chicks receiving this high fat diet.

Methods. The chicks were from a commercial hatchery. They were distributed into groups of 6 chicks each when 1 day old and were kept on the experimental diets for a 4-week period. The chicks were maintained in standard electrically heated batteries with screen wire floors. Feed and water were supplied *ad libitum* and total amount of diet consumed by each group during the experiment was recorded. The chicks were weighed at weekly intervals, and the final weight was the chief criterion of response used in evaluating the results of the experiments. The diets were similar to those used previously(10,11). Diet C29 had the following composition/kg: soybean oil meal 350 g, ground yellow corn 575 g, glucose 10 g, corn oil 5 g, chick salts A 60 g(12), riboflavin 8 mg, vit. D_3 0.02 mg, and 2-methyl-1, 4-naphthoquinone 1 mg. The small amounts of glucose and corn oil served as vehicles for the vitamins. Diet C30 was identical with diet C29 except that 200 g of lard were substituted for an equivalent amount of corn in a kg of diet. The total fat content of diet C29 was approximately 3%, by calculation, and that of C30, 22%. The vit. B_{12} content of each diet was varied in these experiments at levels between 5 and 2,000 γ/kg of diet. For parenteral administration, an aqueous solution of the vitamin was injected subcutaneously in the abdomen

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* Part of these data was presented at 39th Meeting of Fed. of Am. Soc. for Exp. Biol., San Francisco, Apr. 11, 1955.

TABLE I. Effect of Dietary Fat on Growth of Chicks Fed Varying Levels of Vit. B_{12}.

	Diet C29 (3% fat)		Diet C30 (22% fat)	
Vit. B_{12} in diet (γ/kg)	No. sig. experiments/ total No. experiments*	4-wk wt (g)	No. sig. experiments/ total No. experiments*	4-wk wt (g)
0	/6	274 ± 7‡	/12	162 ± 5‡
5	1/2	334 ± 13	0/ 6	191 ± 9
5, inj.†	1/1	318 ± 12	2/ 3	226 ± 10
10	1/1	363 ± 19	7/ 8	259 ± 7
10, inj.†	—	—	3/ 3	257 ± 10
20	1/2	321 ± 11	3/ 3	269 ± 12
50	—	—	4/ 5	282 ± 10
100	3/6	343 ± 7	12/12	292 ± 5
200	—	—	3/ 3	291 ± 14
2000	0/1	326 ± 13	3/ 3	302 ± 8

* No. of experiments in which growth was significantly greater (at 1 or 5% level of probability) than that of the control group receiving no vit. B_{12}. There were 6 chicks per experimental group.

† Vit. B_{12} inj. 3 times weekly in the same quantity as that eaten by respective control groups.

‡ Mean ± S.E.

3 times weekly. The vitamin was injected in the same quantity as the mean amount consumed in the diet by the control chicks since the time of the previous injection. At the end of the 4-week experiments, the chicks were killed by decapitation, the livers were removed, and pooled by groups for vit. B_{12} assay. The analyses were carried out according to the USP XV procedure(13) using *Lactobacillus leichmannii*.

Results. The effect of graded levels of vit. B_{12} upon growth of chicks fed either of the 2 diets may be seen from the data presented in Table I. With diet C29, 5γ vit. B_{12}/kg of diet promoted optimal growth in all tests. In some experiments, the chicks grew maximally even in the absence of dietary vit. B_{12}, so the requirement with this diet always fell between 0 and 5 γ vit. B_{12}.

With diet C30, the growth responses to various levels of vit. B_{12} were markedly different from those with diet C29. As reported previously, growth with this diet was always poor in the absence of vit. B_{12}(10). The 5 γ level failed to improve growth significantly in 6 experiments. With increasing additions of vit. B_{12} up to 50 and 100 γ/kg of diet, growth progressively improved to a maximal level. Considerable variation was observed between experiments in the growth response to vit. B_{12}. Although the details are not presented in Table I, 100 γ vit. B_{12}/kg of diet supported growth that was significantly better than that with 50 γ in 3 of 5 experiments where these two levels were fed (average 4-week weight 304 and 261 g, respectively; $p = < 0.05$). In the 2 other experiments maximal growth was attained at 4 weeks with 50 γ vit. B_{12}. Addition of vit. B_{12} in quantities greater than 100 γ/kg of diet resulted in no further improvement in growth. The requirement under these conditions, therefore, falls between 50 and 100 γ/kg of diet.

From the growth data in Table I, it appears that with the low level of 5 γ vit. B_{12} in diet C30, the injected chicks grew better than the controls receiving the vitamin in the diet. When only the weights of chicks are considered from the three experiments in which the vitamin was both fed and injected, the chicks receiving 5 γ vit. B_{12}/kg of diet averaged 171 g ± 10 and the injected groups averaged 226 g ± 10 ($p = < 0.01$). At the lowest part of the vit. B_{12} growth curve, small amounts of the vitamin resulted in appreciable increments in weight gain; therefore, the slight increase in activity due to injecting the vitamin could be demonstrated at this sub-optimal level. With 10 γ vit. B_{12}/kg of diet C30 and 5 γ in diet C29, no differences in growth were noted between groups of chicks receiving the vitamin by mouth or by injection.

The livers from most of the groups represented in Table I were analyzed for vit. B_{12}. For ease of presentation, the values obtained

TABLE II. Storage of Vit. B_{12} in Livers of Chicks Fed Varying Levels of Vit. B_{12} in Low and High Fat Diets (6 Chicks/Group).

Vit. B_{12} in diet (γ/kg)	Liver wt (g)	Vit. B_{12} in liver		Vit. B_{12} intake, avg/chick/4 wk (γ)	Intake stored in liver (%)
		mγ/g	γ/liver		
Diet C29 (3% fat)					
0	7.6	15	.11	.0	
5	8.2	40	.33	2.95	7
5, inj.*	8.0	50	.40	2.95	10
10	9.0	105	.94	6.35	13
100	8.8	605	5.32	62.40	8
2000	7.3	820	5.98	1108.00	0.5
Diet C30 (22% fat)					
0	3.6	35	.13	.0	
5	5.7	35	.20	1.84	4
5, inj.*	6.4	50	.32	1.84	10
10	7.0	70	.49	5.00	7
50	6.5	160	1.04	19.80	5
75	6.8	265	1.80	31.72	5
100	5.7	440	2.51	42.20	6
200	6.8	540	3.67	888.00	0.4
2000	7.6	780	5.93	912.00	0.6

* Vit. B_{12} inj. 3 times weekly in the same quantity as that eaten by respective control groups.

in one experiment only are presented in Table II. The changes demonstrated by these data are representative of all experiments. In the absence of dietary vit. B_{12}, the amount of vitamin in the liver was essentially the same for the 2 diets. Also, with each diet, increasing levels of vit. B_{12} in the diet resulted in increased concentrations of the vitamin in the liver. This progressive increase in liver stores was more pronounced with diet C29 than with diet C30, because the average 4-week consumption of vit. B_{12} was slightly higher with diet C29 at each level of dietary vit. B_{12}. The proportion of the intake retained in the liver was essentially the same up to the 100 γ level with each diet. In general this percentage was slightly lower with diet C30 than with diet C29. With both diets, the extremely high level of 2 mg vit. B_{12}/kg of diet resulted in very high concentrations in the liver, but this represented a very small proportion of the intake. Similarly, 200 γ/kg of diet C30 resulted in a low rate of storage. The injected vitamin appeared to be stored slightly better than that fed in the diet.

Discussion. Incorporation of 20% fat into a corn-soybean oil meal diet not only increases the severity of a vit. B_{12} deficiency in non-depleted chicks but also increases the requirement for vit. B_{12}. This increase is 10 to 20 times the requirement of control chicks fed the low fat diet (3%) and about 2 to 4 times the requirement reported by others for depleted chicks. The high level of fat did not result in depletion of the animal's chief store of the vitamin, since the content of vit. B_{12} in the liver was similar for chicks fed the high and the low fat diets with no dietary source of vit. B_{12} for 4 weeks. Also, the fat did not prevent the normal absorption of vit. B_{12} from the intestinal tract and its subsequent storage in the liver.

Since vit. B_{12} becomes a critically limiting nutrient when the fat content of this diet is increased, these studies suggest a possible role for vit. B_{12} in the utilization of dietary fat. However, the chicks fed diet C30 did not have any apparent difficulty in metabolizing the large amount of dietary fat, even in the absence of vit. B_{12}. All livers appeared normal upon gross observation; the concentrations of water, total lipid, and phospholipid of the livers fell within normal ranges (unpublished data). Extraction of the feces from chicks fed this diet indicated almost complete absorption of the fat in the diet.

It is possible that the effect of fat upon the chick's requirement for vit. B_{12} is indirect and may be mediated through some other nutrient.

The substitution of 20% lard for an equivalent amount of corn increased the caloric density of the diet by approximately 40% and the ratio of calories to dietary protein by about 50%. The carbohydrate content of the diet was similarly reduced. These altered relationships may have been involved in the increased requirement for vit. B_{12} observed with the high fat diet.

Further discussion of the mechanism by which the high fat diet increased the chick's vit. B_{12} requirement will be considered more appropriately in a subsequent report on the "sparing" effect of certain nutrients upon vit. B_{12} under these conditions.

The graded growth response to varying levels of vit. B_{12} which has been obtained, makes diet C30 suitable for assessing the vit. B_{12} activity of concentrates or analogues for the chick. This diet has proven very useful in our laboratory for this purpose(11). The addition of other nutrients, such as methionine or choline, may alter the chick's response to vit. B_{12}, therefore, this diet cannot be used to evaluate the vit. B_{12} activity of low potency materials.

Summary. Incorporation of 20% fat into a corn-soybean oil meal diet increased ten to twenty-fold the vit. B_{12} requirement of nondepleted chicks for optimal growth at 4 weeks of age. The requirement was between 0 and 5 γ per kg of diet (3% fat) and between 50 and 100 γ with the high fat diet. The high dietary fat did not deplete the chick of its vit. B_{12} liver store or alter the absorption of dietary vit. B_{12} and its subsequent storage in the liver.

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1. Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, 1948, v174, 1047.

2. Lillie, R. J., Denton, C. A., and Bird, H. R, *ibid.*, 1948, v176, 1477.

3. Stokstad, E. L. R., Jukes, T. H., Pierce, J., Page, A. C., Jr., and Franklin, A. L., *ibid.*, 1949, v180, 647.

4. Ott, W. H., *Poultry Sci.*, 1951, v30, 86.

5. Davis, R. L., and Briggs, G. M., *ibid.*, 1951, v30, 628.

6. Miller, R. F., Norris, L. C., and Heuser, G. F., *ibid.*, 1956, v35, 342.

7. Committee on Animal Nutrition, National Research Council, Pub. 301, 1954, 6.

8. Milligan, J. L., Arscott, G. H., and Combs, G. F., *Poultry Sci.*, 1952, v31, 830.

9. Smith, E. L., *Nutrition Abst. and Rev.*, 1951, v20, 795.

10. Spivey, M. R., Briggs, G. M., and Ortiz, L. O., PROC. SOC. EXP. BIOL. AND MED., 1954, v85, 451.

11. Briggs, G. M., and Fox, M. R. Spivey, *ibid*, 1955, v89, 318.

12. Briggs, G. M., Spivey, M. R., Keresztesy, J. C., and Silverman, M., *ibid.*, 1952, v81, 113.

13. *The Pharmacopeia of the United States of America, Fifteenth Rev.*, 1955, 885.

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Effects of Hypoxia on Iron Absorption in Rats. (22798)

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Many studies have shown that gastro-intestinal absorption of iron is increased, in experimental animals(1,2,3) and human subjects(4), during many diverse types of anemia. Granick(5) has suggested that accompanying hypoxia is probably the only common factor capable of increasing iron absorption.

The present experiments were designed to test the hypothesis that hypoxia is capable of increasing iron absorption without the complicating presence of anemia or dietary changes.

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Methods and materials. We used young male rats of the August strain, each weighing about 120 g. The animals were fed exclusively on M.R.C. diet No. 41,[†] food and water being freely allowed. During experiments the animals were housed singly in metabolism cages. For the hypoxia experiments the cages were placed in an airtight glass tank through which a gas mixture, consisting of 15% oxygen and 85% nitrogen, was passed at 3 liters per minute. In the corresponding control experiment air was passed through the tank at the same rate. Radioactive iron (Fe^{59}) was administered by stomach tube while the animal was lightly anaesthetized with ether. The amount of iron administered to each rat was 0.6 μc $Fe^{59}Cl_3$ (less than 1 μg Fe) contained in 0.2 ml acetate buffer at pH 5. The following experiments were performed: 1. Controls: 8 animals were exposed to air at 3 liters flow/minute for 48 hours, then given Fe^{59} and immediately replaced in the same environment for a further 24 hours. 2. 9 animals were exposed to the hypoxic atmosphere for 48 hours, then given Fe^{59} and immediately replaced in the hypoxic atmosphere for a further 24 hours. In the remaining group, animals were kept at normal room atmosphere. 3. 27 control animals were given only Fe^{59}. In each case, feces were collected daily for 6 days. Feces were placed in small glass tubes and their radioactivity assayed by direct counting in a well-type scintillation counter (efficiency for Fe^{59} approximately 10%). Fe^{59} was administered at approximately the same time of day in each experiment, and it was noted that food intake and volume of fecal excretion were normal during experiments.

Results. The results of the 3 experiments are summarized in Table I. There was no significant difference between iron absorption in free air and in the glass tank at 3 liters/minute. However, in Exp. 2, where animals were exposed to 48 hours of hypoxia prior to Fe^{59} administration, there was a significant increase in iron absorption by average of 31%. ($P<0.001$).

TABLE I. Fe^{59} Absorption in Control and Hypoxic Rats.

	No. of animals	Mean absorption, % of administered dose	Range, %
1. Control: air, 3 l/min.	8	24 ± 2.83*	16-40
2. Hypoxia (15% O_2), 3 l/min.	9	55 ± 5.0	37-76
3. Control: free air	27	33 ± 1.54	16-47

* Stand. error of mean.

Throughout these experiments it was found that Fe^{59} excretion was maximal throughout the first 24 hours and thereafter fell off rapidly to negligible amounts after 6 days.

Discussion. These results confirm the hypothesis that hypoxia is capable of increasing absorption of iron from the gastro-intestinal tract without the presence of anemia or dietary changes.

The mechanism by which hypoxia increases gastro-intestinal absorption of iron remains obscure. However, recently it has been shown that hypoxia causes a redistribution of tissue iron(6) and an increase in chromoprotein oxygen transport systems(7,8). Possibly iron is mobilized from the intestinal mucosa for this purpose, under the influence of hypoxic stress; however, these processes probably occur too slowly to account for our results. On the other hand, Granick(9), in a recent review of iron metabolism, has suggested that hypoxia may influence iron absorption by an indirect effect on a humoral mechanism influencing the intestinal mucosa. Undoubtedly, hypoxia is capable of influencing iron metabolism very rapidly. In this respect, the studies of Huff and co-workers(10) are of interest. They found that plasma iron turnover rates and red cell iron renewal rates were increased to approximately twice their normal values after less than 12 hours at high altitude.

Another explanation of these findings is that the hypoxic stimulation of erythropoiesis sets off a humoral mechanism which is capable of causing increased gastro-intestinal iron absorption.

Summary. 1. Experiments with Fe^{59} were carried out to test the hypothesis that hypoxia increases iron absorption. 2. Hypoxia was found to increase iron absorption by approximately one-third.

[†] Medical Research Council diet No. 41 is a balanced diet with adequate mineral content.

1. Stewart, W. B., Vassar, P. S., and Stone, R. S., *J. Clin. Invest.*, 1953, v32, 1225.

2. Copp, D. H., and Greenberg, D. M., *J. Biol. Chem.*, 1946, v164, 377.

3. Hahn, P. F., Bale, W. F., Ross, J. F., Balfour, W. M., and Whipple, G. H., *J. Exp. Med.*, 1943, v78, 169.

4. Dubach, R., Callender, S. T. E., and Moore, C. V., *Blood*, 1948, v3, 526.

5. Granick, S., *Bull. N. Y. Acad. Med.*, 1949, v25, 403.

6. Keller, J. G., *Am. J. Physiol.*, 1955, v183, 633.

7. Criscuolo, D., Clark, R. T., and Mefford, R. B., *ibid.*, 1955, v180, 215.

8. Vannotti, A., *Schweiz. med. Wschr.*, 1946, v76, 899.

9. Granick, S., *Bull. N. Y. Acad. Med.*, 1954, v30, 81.

10. Huff, R. L., Lawrence, J. H., Siri, W. E., Wasserman, L. R., and Hennessy, T. G., *Medicine*, 1951, v30, 197.

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A Simple Method of Cutting Split Thickness Skin Grafts from Small Animals. (22799)

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The great increase in interest in homografting and especially in that of skin homografting prompts us to report what we have found to be a simple and reliable method of obtaining split thickness grafts from the rabbit and rat. Full thickness, pinch, and split thickness grafts all have their advocates and peculiar advantages. The split thickness graft, however, has been especially useful in our work for several reasons. 1) It has a high initial take (we have not yet had a failure). 2) Its hairlessness, softness and thinness make viability easy to evaluate, and also make it ideal for use in millipore chamber technics. 3) It requires no dressing of any kind.

Methods heretofore mentioned in the literature are either too cumbersome or require expensive equipment such as the Brown Electro dermatome. We have found the following method to be simple, rapid, and dependable. The equipment required is inexpensive and can be used by relatively inexperienced persons.

Method. In essence the skin to be used is stretched tightly over a flat block by means of a special clamp and a graft is cut freehanded.

In detail: 1) Donors are clipped closely and widely over the donor site and the remaining stubble is epilated with Barium Sulphide and corn starch paste. The donors are then kept for 1-3 days at which time a fine stubble of new hair growth will indicate the anlagen phase of the skin or hair growth cycle. 2) Under barbiturate anesthesia an incision is made at one end of the donor area and the skin and panniculus carnosa is elevated from the underlying fascia by blunt dissection. 3) The specially shaped block (Fig. 1) is introduced into the space thus formed and the clamp is applied so as to hold the skin stretched tightly over the block. 4) At this point it is advantageous to make any desired identifying marks by pricking the skin with a #24 needle dipped in India ink (Fig. 2). 5) The graft can now be cut easily with a straight razor held freehand. The clamp serves as a handle to steady the skin. Grafts of as much as 60 sq cm can be cut in a single sheet from a 300 g rat (Fig. 2). 6) The clamp is removed, the block withdrawn and the incision closed with Michel clips or may be sutured as desired. 7) The grafts may be applied to the recipient bed by any method. We have found Michel clips to be a very rapid and entirely satisfactory way of securing the graft (Fig. 3). 8) By placing the

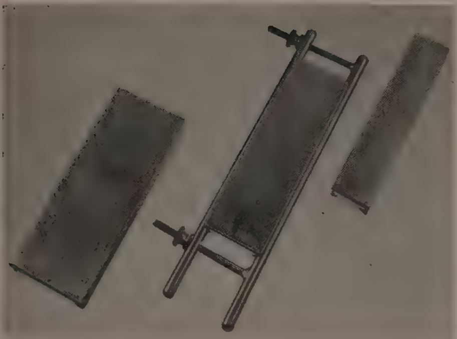

FIG. 1. Stainless steel clamp and three Duraluminum blocks of different widths which it will fit.

grafts on the dorsum of the recipients and isolating the animals for the first 48 hours we have not found it necessary to use any type of protective or pressure dressing.

In 25 rats autografted with 4 x 10 cm grafts the only slough which occurred was in the small points of pressure from the clips. Otherwise, all grafts could be considered 100% takes. Infection or hematoma formation under the grafts has not occurred at any time even in animals severely debilitated by toxic medications. No attempt at sterile technic has been made.

Summary. The details of a new method of cutting split thickness skin grafts from small animals are reported with a discussion of some of the advantages.

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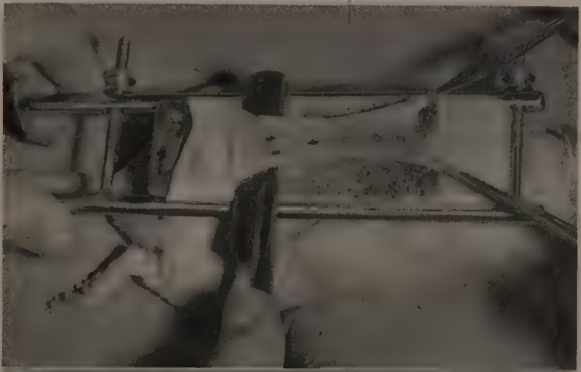

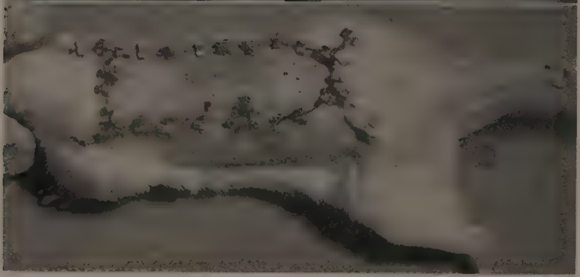

FIG. 2 (top). Clamp in place and graft being cut. Notice identifying tattoos.
FIG. 3 (bottom). Homograft on 6th postoperative day. No dressing was used. The granulating area represents a button hole in the graft. The dark mottling in the center of the graft was reddish-purple at the time of the photograph, and was the first area of slough.

Effect of Extracts of Developing Muscle on Tissue Culture.* (22800)

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Gaillard(1) has shown that the growth promoting activity of extracts of whole chick embryos varies depending upon the age of the extracted embryos. From these experiments little could be concluded as to a possible correlation of growth promoting activity and the state of proliferation and differentiation of the donor tissue since whole embryo extracts include components of tissues at widely differing stages of development. Examination of extracts from adult tissues with different rates of cell proliferation did not indicate that proliferative activity in a tissue and growth promoting activity of its extracts are related (2). As a result of a more systematic study of the cytological and chemical changes in developing leg muscle of the chick(3,4), data have become available which invite a reconsideration of the relationships of the developmental stage in a single tissue and the growth promoting activity of its extracts. The results of tests of the growth promoting activity of extracts from developing chick leg musculature are reported in this paper.

Materials and methods. The muscle extracts to be tested were prepared by carefully dissecting the muscle tissue from the legs and passing it through a Latapie grinder. For each gram of pulp 1 ml of Pannett and Compton (P.C.) solution(5) was added. The mixture was immediately centrifuged (2,000 x g) and the supernatant decanted (designated as 50% extract). The tested extracts were obtained from leg muscle of the 12 day and 19 day chick embryo, and the 10 day and one-year chick because the growth characteristics of muscle tissue differ qualitatively and quantitatively at these stages of development. The mean protein concentration in muscle extracts, determined following Lowry's procedure(6), corresponds to 10.3, 19.7, 20.9 and 25.4 mg protein per ml of 25% extract, respectively for the extracts from the 4 ages given above. The embryo extract used for the first 4 passages was prepared in a similar way using the entire 12 day embryo as starting material. Coverslip cultures derived from leg muscle explants of 12 day embryos were used to assay the growth stimulating activity of muscle extracts. During 4 passages the cells were grown in a clot medium consisting of 2 drops of chicken plasma and 2 drops of 25% embryo extract with transfer to new medium every other day. At the fifth passage the whole embryo extract in the medium was replaced by P. C. solution (controls) or by muscle extracts of the desired concentration and donor age. Twelve hours and 48 hours after the last transfer the surface area of the cultures was recorded with a camera lucida, measured with a planimeter, and the relative increase computed according to Ebeling(7).

Results. The reported measurements of cell culture areas include both cell migration and cell proliferation in unknown proportions. Therefore, the term "growth" can be used in the context of this paper only with the reservation of this incomplete definition. Analysis of the results listed in Table I shows that addition of 0.125% extract from muscle of any of the 4 tested stages of development does not stimulate significantly[‡] the "growth" of the test cultures. With extracts from 12 day embryos maximal activity is obtained only with the highest concentration tested (12.5%) whereas the extracts from muscle of the 12 day embryo and the 10 day chick give the same maximal "growth" stimulation with a 10 times lower concentration. The extracts of the one-year-old chick muscle remain sig-

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[‡] Significance is attributed to differences with a statistical probability of chance occurrence of less than 1%.

TABLE I. Compiled Results of Clot Culture Experiments.

Age of extract donor	Conc. of extract in medium, %	Mean relative increase,* mm²	No. of samples
12-day embryo	.125	3.53 ± .5	15
	1.25	6.74 ± .4	47
	12.5	10.40 ± .5	53
19-day embryo	.125	3.27 ± .4	31
	1.25	11.87 ±1.1	29
	12.5	10.90 ± .5	42
10-day hatched chick	.125	5.14 ± .7	17
	1.25	11.80 ±1.0	44
	12.5	8.52 ± .6	53
1-year-old adult	.125	3.48 ± .6	10
	1.25	5.63 ± .4	33
	12.5	7.46 ± .4	27
Controls	.00	4.71 ± .6	25

* $\frac{\text{Area}_2\ (48\ \text{hr}) - \text{Area}_1\ (12\ \text{hr})}{\text{Area}_1\ (12\ \text{hr})}$ ± S.E. of mean.

nificantly below maximal activity even in the highest concentration. The decrease in the "growth" stimulation with the 19 day embryo extract (12.5%) is statistically insignificant, while the drop in the activity of the 10 day chick extract (12.5%) is significant.

Since the 19 day embryo extracts and the 10 day chick extracts give maximum "growth" promoting activity with the 1.25% extract concentration, the accumulation of the "growth" promoting substances apparently reaches an optimum during the last days of the pre-hatching and the first days of the post-hatching periods. Whether the extracts from the 19 day embryo or the 10 day chick are more active can not be conclusively stated at the present time. A reversal in "growth" promoting activity by excessively high concentrations of tissue extracts has been observed repeatedly(1,8) but its significance has been questioned(9) because of the frequently occurring liquefaction of the media. In the present series of experiments, cultures with liquefied plasma clot were not measured and, therefore, the marked decrease in growth stimulation with the highest concentration of 10 day chick extract may indicate a greater excess of "growth" promoting substances than in the muscle extracts from other stages of development.

Discussion. The period from the 12th to the 19th day of development of the chick embryo is a phase of development in which the rate of proliferation of the leg muscle declines. Correspondingly, total accumulation of DNA, as an index of cell proliferation, was found to come practically to a standstill on the 18th day of development(4,10). On the other hand, formation of specific cell proteins such as collagen (fibroblasts)(11) or of myosin(12) (myoblasts) takes place at a rapid rate during the 12th to the 18th day period of development and myosin production continues at a considerable rate for several weeks after this date. It is apparent that a maximal "growth" promoting activity of muscle extracts is observed when cell proliferation declines sharply and formation of specific cell proteins becomes the predominant growth component.

Summary. 1. Determinations were carried out of the effect of extracts from developing chick leg muscle on the increase in area of coverslip cultures derived from chick leg muscle cells. 2. Maximal stimulation with the lowest extract concentration was obtained with extracts from muscle of the 19 day embryo and the 10 day chick. Extracts from the 12 day embryo and the one-year chick were less effective. 3. The activity of the muscle extracts was found to be unrelated to the rate of cell proliferation in chick muscle tissue.

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1. Gaillard, P. J., *Protoplasma,* 1935, v23, 145.

2. Hoffman, R. S., Tenenbaum, E., and Doljanski, L., *Growth,* 1940, v4, 207.

3. Herrmann, H., *Ann. N. Y. Acad. Sci.,* 1952, v55, 99.

4. Herrmann, H., White, B. N., and Cooper, M., *J. Cell. Comp. Physiol.,* in press.

5. Pannett, C. A., and Compton, A., *Lancet,* 1924, v206, 381-384.

6. Lowry, Oliver H., Rosebrough, Nira J., Earr, A. Lewis, and Randall, Rose J., *J. Biol. Chem.,* 1951, v193, 265.

7. Ebeling, Albert H., *J. Exp. Med.,* 1921, v34, 231.

8. Parker, R. C., *Arch. Exp. Zellfschg.,* 1929, v8, 340.

9. Willmer, E. N., and Jacoby, F., *J. Exp. Biol.,* 1936, v13, 237.

10. Konigsberg, I. R., and Herrmann, H., *Arch. Biochem. Biophys.,* 1955, v55, 534.

11. Herrmann, Heinz, and Barry, Shirley R., *ibid.,* 1955, v55, 526.

12. Czapo, A., and Herrmann, Heinz, *Am. J. Physiol.,* 1951, v165, 701.

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Effect of Hypothermia upon Induced Bacteremia.* (22801)

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In this laboratory observations have been made upon dogs maintained hypothermic for prolonged periods of time(1). The cause of death in some of these animals during the period of cooling and following rewarming remains unexplained. In a study by Parkins, Ben, and Vars(2), upon the effect of differential cooling in fatal ischemic shock, it was shown that a greater number of animals survived preferential cooling of the intestine than primary cooling of the liver. This led Rhoads *et al.*(3), to suggest that intestinal bacteria or their endotoxins might be a factor contributing to the poor results obtained from paramount cooling of the liver. Overton and DeBakey observed(4) that anesthesia and hypothermia, with autonomic blocking agents, was followed by a mortality of 10% when antibacterial therapy was not employed. That a bacterial factor exists in hemorrhagic shock in normothermic dogs is indicated by the experiments of Schweinburg, Frank, and Fine(5). Whether such a factor is present in the hypotension of prolonged hypothermia and subsequent rewarming has not been ascertained. The purpose of this paper is to present data showing (1) that hypothermia even after many hours does not promote a state of bacteremia, and (2) that the hypothermic animal is still capable of removing pathogenic organisms injected into its blood stream.

Methods. Healthy mongrel dogs of both sexes weighing 6.8 to 20.4 kg were used. The technic of cooling used in this laboratory has been adequately described in detail(1). Briefly, animals anesthetized with ether, were placed in a cold water bath at 1°C until the rectal temperature was 28°-29°C. Following removal from bath they were maintained at 22°-24°C in air-conditioned room. Rewarming was accomplished by placing dogs in a 40°C water bath where they were kept until rectal temperature was 28°-29°C. Following drying with towels, animals were placed in warm room where they returned to normothermic levels. Blood samples were obtained by percutaneous puncture of jugular vein. Sterile conditions were maintained during periods of sampling and culturing. To determine presence or absence of bacteremia during hypothermia and during rewarming, blood samples were obtained just prior to cooling following 6 or 12 hours of hypothermia and after 2 to 8 hours of rewarming. These samples were then incubated 48 hours at 37°C in brain heart infusion for determination of aerobic growth and chopped meat broth for anaerobic growth. Transfers were made from thioglycolate broth to blood agar plates and Levine E.M.B. plates and observed for growth and identification of organisms. Transfers from chopped meat broth to blood agar plates were incubated in environment of CO_2 at 37°C. The effect of hypothermia and rewarming on bacterial clearance was determined by intravenous injection of saline suspension of 24 hour culture of *E. coli* and *A. aerogenes* which had been prepared previously from cultures of stools of the animals. All dogs were inoculated with 5-50 billion bacteria. The number of organisms introduced was determined by nephelometry. The first sample of blood, 2 cc, was taken 10 minutes after injection and mixed with liquid agar, plated in duplicate and incubated at 37°C. Succeeding samples were drawn at 6, 12, 18, and 24 hours. Following withdrawal of the 24 hour sample, rewarming of the animal was instituted. Additional 2 cc samples were taken and plated 6, 12, and 24 hours after removal of animal from the warm water

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[†] John and Mary Markle Scholar.

TABLE I. Blood Clearance of 5-50 Billion Bacteria Injected into 5 Normothermic Dogs.

No. of colonies/ml of blood				
10 min.	6 hr	12 hr	18 hr	24 hr
2,750	9	3	7	0
5,025	0	1	3	0
2,750	23	3	4	0
4,775	5	4	3	0
5,770	4	2	3	0

bath. After a 48 hour incubation period at 37°C, bacterial colony counts were made on a Quebec-Colony Counter. An average of the number of colonies on duplicate plates was made and results expressed as number of colonies per cc of blood.

Results. Twelve animals were cooled either 6 or 12 hours and then rewarmed. All of these animals maintained the sterility of their blood both during the period of hypothermia and following rewarming. In spite of this, 2 of the 6 animals cooled for 12 hours died 5 and 8 hours following rewarming.

To determine the effect of hypothermia and rewarming on the capacity to destroy bacteria, 5 normothermic and 13 hypothermic dogs were inoculated with 5-50 billion bacteria (*E. coli* and *A. aerogenes*). The normothermic dogs (Table I) revealed no ill effects and completely cleared their blood streams of bacteria in 24 hours. Hypothermic animals were divided into 2 groups (Table II). Group I was injected with bacteria when their temperatures were stabilized at 23 ± 0.6°C. Group II was injected 6 hours after hypothermia had been maintained at 23 ± 0.6°C. In the first group all animals except one had positive blood cultures after 24 hours of hypothermia. Following rewarming all animals in this group demonstrated negative blood cultures within 24 hours. Three of the dogs died 12, 42, and 44 hours following rewarming. All of these animals had negative blood cultures before death and at autopsy no apparent cause of death could be ascertained. In the second group, where bacteria had been injected after 6 hours of hypothermia at 23°C the ability to clear organisms appears to be about the same as in the first group. One animal in this group died during hypothermia although a blood culture taken previous to death revealed no growth. Three other dogs died following rewarming. Only one of these demonstrated a bacteremia. In this animal, however, there was a decline from 7000 colonies per cc to 3 colonies per cc during the course of the experiment.

Discussion. Although animals maintained hypothermic for long periods of time did not clear bacteria from the blood as efficiently as the normothermic dogs, phagocytosis was not abolished. Samples of blood from hypothermic dogs, 6 hours subsequent to injection of bacteria, indicate a 99% phagocytosis in all but one animal. In all instances, the 12, 18, and 24 hour samples from cooled animals showed the blood to be free of 99% of the initially injected bacteria. Recently it has

TABLE II. Blood Clearance of 5-50 Billion Bacteria Injected into 2 Groups of Dogs during Hypothermia and after Rewarming.

	No. of colonies/ml of blood								
	During hypothermia					After rewarming			
	10 min.	6 hr	12 hr	18 hr	24 hr	6 hr	12 hr	24 hr	Remarks
Group I	7,900	3	3	7	2	0	0	0	Died
	4,650	2	0	3	0	0	0	0	Survived
	3,775	13	9	6	19	5	4	0	"
	4,450	12	11	1	105	50	5	0	Died
	12,500	4	23	20	14	3	0		"
Group II	2,800	2	0						Died
	9,500	21	50	10	7	3	0		"
	7,000	1,000	27	20	14	7	3		"
	3,000	15	9	3	3	0	0	0	Survived
	9,200	42	60	13	4	2	0	0	"
	8,500	42	13	5	1	0	0	0	"
	8,000	2	2	6	0	0	0		Died

Group I: Bacteria inj. when temp. stabilized at 23.0 ± 0.6°C. Group II: Bacteria inj. 6 hr after hypothermia at 23.0 ± 0.6°C.

been demonstrated that there is an 86% reduction in the leucocyte count in dogs cooled to the range of 18°-26°C for a hundred minutes(6) and that the leukopenia disappears within a half hour of rewarming(7). *In vitro* studies of temperature relations in phagocytosis by Harmon and associates(8) showed that the phagocytic powers of the polymorphonuclear leucocytes decreased with lowered temperatures. Since the experiments in this report encompass the time interval reported by Helmsworth(6), a leukopenia should be expected at the time of the injection of the bacteria. It would thus seem that the paucity of leucocytes and the decline of the phagocytic powers of the remaining leucocytes could not account for the 99% removal of the injected bacteria as seen here. It is reasonable to assume that the reticulo-endothelial system of the liver and spleen assumes the major role in phagocytosis under these conditions. Manwaring(9) and Cannon(10), on a basis of blood flow, availability of macrophages and leucocytes, and mechanical conditions favoring filtration, felt that the liver and spleen were the principal phagocytic centers. Fisher *et al.*(11) showed that there was a progressive decrease in hepatic blood flow as the hours of hypothermia were extended and that on rewarming there was a prompt return to control levels. The removal of bacteria from the circulation as reported here would seem to parallel these changes in blood flow. A trapping and intermittent release of contaminated blood as well as the decrease in hepatic blood flow may account for the incomplete phagocytosis which occurs during the hypothermia. It would seem that it is impossible to relate the cause of death in rewarming following prolonged hypothermia to a bacterial factor or at least a bacteremia. Although 7 out of 12 animals into whose circulation bacteria were injected died after being rewarmed, in only one of these was there a positive blood culture.

Conclusions. 1. Dogs cooled to 23°C 6 or 12 hours and then rewarmed, maintained sterility of blood during cooling and following rewarming, suggesting that the gastrointestinal tract and other bacterial foci maintained their integrity for at least 12 hours of hypothermia. 2. Hypothermic and normothermic animals intravenously inoculated with bacterial suspension were able to rid blood within 6 hours of 99% of the injected pathogens. Within 24 hours after injection of cultures of *A. aerogenes* and *E. coli*, normothermic animals cleared the blood completely of bacteria while dogs maintained hypothermic did not. However, within 12-24 hours after rewarming only 1 of 11 hypothermic animals had a bacteremia. 3. Inability to survive following prolonged hypothermia does not seem to be related to bacteremia.

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1. Fisher, B., Russ, C., Fedor, E. J., Wilde, R., Engstrom, P., Happel, J., and Prendergast, P., *A. M. A. Arch. Surg.*, 1955, v71, 431.

2. Parkins, W. M., Ben, M., and Vars, H. M., *Surgery*, 1955, v38, 38.

3. Rhoads, J., Varn, W., Parkins, W. M., Ben, M., and Vars, H. M., *Surg. Clin. N. A.*, 1955, v35, 1585.

4. Overton, R. C., and DeBakey, M. E., *Ann. Surg.*, 1956, v143, 439.

5. Schweinburg, F. B., Frank, H. A., and Fine, J., *Am. J. Physiol.*, 1954, v179, 532.

6. Helmsworth, J. A., Stiles, W. J., and Elstun, W., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 474.

7. Villalobos, T. J., Adelson, E., and Brila, T. G., *ibid.*, 1955, v89, 192.

8. Harmon, D. R., Zarafonetis, C., and Clark, P F., *J. Bact.*, 1946, v52, 337.

9. Manwaring, W. H., and Fritschen, W., *J. Immunol.*, 1923, v8, 83.

10. Cannon, P. R., Sullivan, F. L., and Neckermann, E. F., *J. Exp. Med.*, 1932, v55, 121.

11. Fisher, B., Fedor, E. J., Lee, S. H., Weitzel, W. K., Selker, R., and Russ, C., *Surgery*, 1956.

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Urinary Excretion of 5-Hydroxyindole Acetic Acid, A Serotonin Metabolite, in Hypertensive Renal-Vascular Disease.* (22802)

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The recent literature has called attention to the syndrome of metastatic carcinoid, manifested by vascular cutaneous flushing, respiratory distress and chronic diarrhea associated with right sided valvular disease of the heart (1-2). The work of Lembeck(3) and Udenfriend(4,6) has resulted in the demonstration of high concentrations of 5-hydroxytryptamine (5HTA, serotonin) in carcinoid tumors and the detection of the 5HTA metabolite, 5-hydroxyindole acetic acid (5HIAA), in urine(5). Sjoerdsma and Udenfriend(6) in their excellent study of patients with the "carcinoid syndrome" have demonstrated high titers of 5HTA in blood and high concentrations of 5HIAA in urine of such patients.

Several authors(2,10) have called attention to the fibrous tissue proliferation present in many of the patients with long standing "carcinoidosis." They have particularly noted the unusual and extensive distribution about the right heart (fibrous thickening of the endocardium with pulmonic and/or tricuspid stenosis) as well as fibrous tissue proliferation in the pelvis and about the abdominal viscera. In some instances evidence of previous inflammatory lesions was noted as indicated by the microscopic description of new blood vessels and occasional round cell infiltration. Because of the vasomotor disturbances, fibrous tissue proliferation and the apparent role of 5HTA metabolism in patients with the "carcinoid syndrome," it seemed worthwhile to study 5HTA's metabolite, 5HIAA in patients demonstrating the severe arterial lesions, which are found in the acute fibrinoid necrosis of malignant hypertension(7,8).

Methods and materials. Twenty-four hour urine specimens were obtained in all instances mentioned in this report with 10 cc of toluene as a preservative. 5HIAA was determined by the procedure of Udenfriend, Titus and Weissbach(5). Normals were obtained on 15 adults and 2 children. Six patients were classified as malignant hypertension because they demonstrated necrotizing arteriolitis and/or papilledema. One of these (J.S., wt. 16 kg) had received Serpasil, 17.80 mg orally in 48 days after which her initial urinary 5HIAA was determined. She then received 19.9 mg of Serpasil I.M. in 8 days with subsequent 5HIAA determinations. Also included are 2 patients presenting the late phase of chronic glomerular nephritis and 2 patients on whom 5HIAA exertion was determined after removal of early carcinoid tumors of the intestinal tract. There has been no reference in the literature concerning the effect of proteinuria on the determination of 5HIAA. The following experiment was performed to rule out the possibility that protein might interfere, since all our patients with hypertension and diminished excretion of 5HIAA had proteinuria. Aliquots of urine from patients with proteinuria (5 to 10 g/day) were analyzed and mixtures were made with known normal urines.

The *results* are summarized in Table I. The average daily excretion of 5HIAA in normal adults was 4.4 mg (ranges 2.3 to 6.3) and for 2 normal children 2.7 and 3.4 mg. Low levels (less than 2.) were found in 3 patients with malignant hypertension and 2 patients with advanced changes of chronic glomerular nephritis. One patient (E.P.) with a markedly elevated blood urea nitrogen (81 mg %) had urinary 5HIAA levels in the normal range. Sjoerdsma(9) and his group call attention to low values in patients with collagen vascular disease (lupus erythematous disseminatus). No mention is made of renal involvement in these patients. Conceivably the low values were in patients with renal vascular involvement. Moreover these investigators confined their description of "low val-

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TABLE I. Urinary Excretion of 5-Hydroxyindole Acetic Acid.

Diagnosis		Comment	No. of patients	Age	Bun. at time of analysis (mg %)	Avg 24 hr urine vol (cc)	Urinary excretion (mg/24 hr)
Normal subjects	Adults		15	18 to 56		1410	2.3 to 6.3, avg 4.4
	Children		2	11		1140	2.7, 3.4
Malignant hypertension	C.L.	Papilledema; autopsy; necrotizing arteriolitis in kidney	1	53	27	1120	1.08
	E.P.	Papilledema	1	40	81	2800	5.4
	E.S.	Papilledema; autopsy; necrotizing arteriolitis of kidney with minimal fibrosis and lymphocytic infiltration	1	29	48	900	2.5
	J.D.	Papilledema; renal biopsy, chronic pyelonephritis with severe arteriolar sclerosis	1	38	38	785	4.8
	G.R.	Papilledema; autopsy, severe arteriolar sclerosis with no inflammatory reaction	1	37	108	200	0.5
	J.S.	Papilledema; autopsy, chronic pyelonephritis with necrotizing arteriolitis	1	7	104	653	1.5 after 17.8 mg Serpasil
					72	797	1.7 } after 1.9 mg Serpasil I.M.
					104	715	0.96 }
Chronic glomerular nephritis	M.S.		1	16	94	1970	1.6
					100	1455	1.9
	W.H.		1	5	88	410	1.4
					20	800	1.2
Carcinoid	E.C.		1	59			2.4, 2.8, 4.0
	S.W.		1	27			3.2

ues" to patients with "collagen vascular disease," while disregarding normal to high levels in such allied collagen disorders as rheumatic fever, rheumatic heart disease and scleroderma (ranges 5.1 to 7.1 mg). Further recording of 5HIAA excretion in patients with proliferative arterial lesions with and without renal involvement will be necessary before any concrete conclusion may be drawn. It must be noted that proteinuric renal disease itself might possibly lead to diminished 5HIAA excretion, although the proteinuria *per se* does not interfere with the determination as indicated by an 82 to 100% recovery of 5HIAA in mixtures of normal urine and the urine from patients with proteinuria.

The reported low values(9) in several cases of malignant disease might also be attributable to concurrent vascular renal disease.

The low excretion of 5HIAA in patients with hypertension might be due either to a poor amine oxidase activity of the diseased kidney, or, more possibly to a retention of serotonin in the blood. These possibilities are presently under investigation in our laboratory.

The 2 patients with early carcinoid tumors excreted normal amounts of 5HIAA, analysis being performed 2-3 weeks after removal of the tumors. Patient S.W. had a small (.5 cm diameter) rectal polyp removed which on microscopic examination revealed a well circumscribed carcinoid tumor. Patient E.C. presented with acute intestinal obstruction, relieved by resection of 45 cm of ileum containing a carcinoid tumor (5 x 6 cm) with metastasis to the accompanying regional lymph nodes. No liver involvement was noted and there were no associated cardio-vascular abnormalities other than fluctuating blood pressure of 190/110 to 160/100, soft aortic systolic murmur, and electrocardiographic changes suggesting diffuse non-specific myocardial disturbance.

Summary. The urinary excretion of 5-hydroxyindole acetic acid in patients with malignant hypertension and chronic glomerular nephritis is reported. Low levels were recorded for 3 patients exhibiting the malignant phase of hypertensive cardiovascular disease and in each patient with chronic glomerular nephritis. All patients exhibited extensive renal involvement and 5 of the 6 patients with malignant hypertension had severe arterial lesions. The 2 patients on whom determinations were made after removal of early carcinoid tumors did not show abnormal urinary 5-hydroxyindole acetic acid levels. Recovery studies show that proteinuria does not interfere with the determination of 5HIAA in the urine.

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1. Thorson, A., Biorek, G., Bjorkman, G., and Waldenstrum, J., *Am. Heart J.*, 1954, v47, 795.

2. Waldenstrum, J., and Ljungberg, E., *Acta. Med. Scandinav.*, 1955, v152, 293.

3. Lembeck, F., *Nature*, 1953, v172, 910; Ratzenhofer, M., and Lembeck, F., *Z. F. Krebsforsch.*, 1954, v60, 169.

4. Udenfriend, S., Weissbach, H., and Clark, C. T., *J. Biol. Chem.*, 1955, v215, 337.

5. Udenfriend, S., Titus, E., and Weissbach, H., *ibid.*, 1955, v216, 499.

6. Sjoerdsma, A., Weissbach, H., and Udenfriend, S., *Am. J. Med.*, 1956, v20, 520.

7. Zeek, P. A., *A. J. Clin. Path.*, 1952, v22, 777.

8. Pickering, G. W., *High Blood Pressure*, Grune & Stratton, N. Y., 1955, p91, 235-240.

9. Haverbach, B. J., Sjoerdsma, A., and Terry, L. L., *N. Eng. J. M.*, 1956, v255, 270.

10. Mattingly, T. W., Sjoerdsma, A., *Modern Concepts Cardiovascular Disease* (Am. Heart Assn.), 1956, v25, 337.

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Distribution of C^{14}-Labeled Aminoacetonitrile in Tissues of Rat, Metabolism and Mode of Elimination.* (22803)

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Several recent studies(1,2,3) have dealt with the effects of aminonitriles on rats. The remarkable ability of these substances to produce the characteristic skeletal lesions of lathyrism in young animals suggests the investigation of the metabolism of the aminonitriles, with the aid of the isotopic labeling technic. Very recently isotopically-labeled β-amino-proprionitrile has been reported synthesized and a study of its metabolism begun (4). For the present work, aminoacetonitrile (AAN) labeled with C^{14} in its cyanide group was employed. It has been previously established(1) that this compound resembles closely in its action, but is more potent than the higher homolog, β-aminoproprionitrile, or β-(γ-L-glutamylamino)-proprionitrile, the active principle of Lathyrus peas. Besides determining the distribution of the radioactivity in the animal organism and the modes of elimination, experiments were designed to explore the possible incorporation of the cyanide carbon atom into major body constituents, including protein, lipid, and carbohydrate. One possible route would be via hydrolysis of the cyanide radical to yield a carboxyl group, *i.e.* the transformation of AAN into glycine.

Methods. Synthesis of $(H_2N\text{-}CH_2C^{14}N)_2H_2SO_4$. The following modification was employed of the method given in Organic Syntheses(5): A solution of NaCN (1 g, 20 mM, containing 1 millicurie of C^{14}) in 1.7 ml water was added slowly to a mixture of 1.1 g of NH_4Cl (20 mM) and 3 ml of 37% formaldehyde (40 mM). When half the NaCN had been added. 0.76 ml of acetic acid was added at such a rate that both the addition of the remaining cyanide and of the acid ended at the same time. The stirring was continued for 30 minutes, and the methyleneiminoacetonitrile was filtered off; yield, 0.70 g (51.5%). The methyleneiminoacetonitrile was treated with a solution of 1 g of 96% H_2SO_4 in 5 ml of ethanol, and allowed to stand over night. The yield of crystalline aminoacetonitrile hydrogen sulfate was 0.91 g or 43% based on NaCN. The recrystallized product melted at 155-7° with decomposition. Its nitrogen content, 25.9%, was lower than theory (26.65), suggesting that a small proportion of the molecules were in the form: $(H_2NCH_2C^{14}N)$ H_2SO_4. Female weanling Sprague-Dawley rats (45-50 g) were given AAN-C^{14} by either: (1) A single IP injection of a neutralized solution of the sulfate salt, or (2) Feeding ground Purina Chow containing 0.01% AAN-C^{14} plus 0.09% non-isotopic AAN for a number of days.† After stated intervals of time, animals were anesthetized, and blood samples taken by heart puncture. The tissues were rapidly removed, weighed, and homogenized with 20 parts of water. Aliquots of each homogenate were dried on steel planchets, and radioactivity measured with a flow-gas Geiger counter. All values were corrected for self-absorption. Subsequently protein was isolated(5) from the tissue homogenates and its C^{14} content determined. In one instance liver protein was hydrolyzed and the radioactivity of its glycine and serine determined(6). Also liver glycogen and lipid were isolated(7) and their radioactivity measured. In other experiments, rats were maintained in metabolic cages for the collection of respiratory CO_2 and urine(8). In studying the forms in which the C^{14} was present in the urine and in the tissues, extensive use was made of the "carrier" method, with 3 to 4 recrystallizations of the isolated substance. In employing this technic, AAN was precipitated by adding alcohol to urine and tissue extracts; urinary urea was isolated by the xanthydrol method(9), creatinine as the picrate(10), and allantoin via the silver and mercury salts(11). In one experiment sodium benzoate was injected together with AAN-C^{14}, and hippuric acid subsequently isolated from the urine(12). Lastly, the procedure of Meyer and Smyth(13) for chondroitin sulfate was employed for the isolation of a polysaccharide fraction‡ from the enlarged epiphyseal cartilage plates of rats maintained on diets containing AAN-C^{14}.

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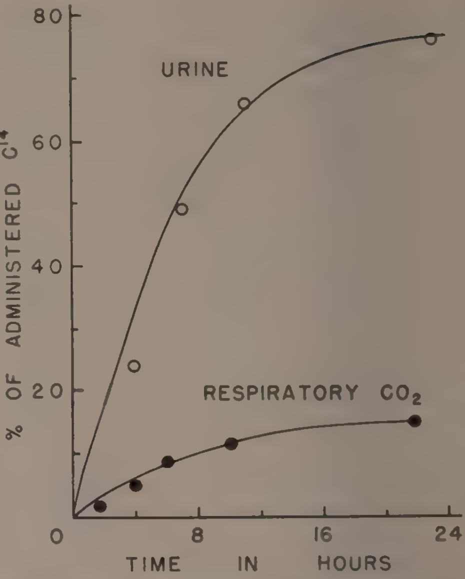

FIG. 1. Rates of elimination of C^{14} in urine and in expired air, following IP inj. of 2 mg AAN-C^{14}. The values are for a group of 6 rats maintained in a common metabolic cage.

Results. Fig. 1 shows that AAN was catabolized to a significant extent, since 15% of the administered C^{14} was found in respiratory CO_2 in 22 hours. Almost 80% of the radioactivity appeared in the urine in this time interval. Further analysis of this same urine, and of urine from rats on a diet containing AAN-C^{14}, revealed that free AAN it-

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† Amounts of nitrile are expressed in terms of free AAN, as calculated from quantity of sulfate employed.

‡ This preparation differed from chondroitin sulfuric acid in its lower mobility and more diffuse pattern in paper electrophoresis at pH 8.9. It was sulfated, as indicated by metachromatic staining with toluidine blue. (Kindly performed by Drs. R. D. Montgomery and T. R. Sawyer).

TABLE I. Distribution of Isotope among Urinary Constituents.

Source of urine*	% of C^{14} retained by carrier AAN	 Urea	 Creatinine	 Allantoin
Rats inj. with 2 mg AAN-C^{14}, urine collected first 4 hr	15	8	3.0	2.1
Same, 4-7 hr urine	18	9	3.5	1.8
Rats on stock diet containing 0.1% AAN-C^{14}, urine collected 6th day	16	12	2.2	1.5

* Pooled urine of 6 rats.

self accounted for only a minor part of the C^{14} eliminated by this route (Table I). The moderate radioactivity in the isolated urea agrees with the finding for respiratory CO_2 The low, but significant isotopic values for creatinine and allantoin suggest that a certain degree of conversion of AAN-C^{14} to glycine-C^{14} occurred, since glycine is a precursor of both these compounds. Further confirmation of this pathway was obtained by injecting a mixture of 1.3 mg AAN-C^{14} and 25 mg sodium benzoate into each of 2 rats. The hippuric acid isolated from the pooled urine (collected over an 18 hour period) accounted for 1.2% of the total administered C^{14}.

Some 60-70% of the C^{14} of urine was not characterized in the present study.§ Schilling and coworkers(4) find that the C^{14} of $NH_2CH_2CH_2C^{14}N$ is likewise chiefly eliminated in the urine of rats, in an altered form. However, they state that the main excretory product "appears to be a volatile, ether-soluble, basic or neutral substance." No volatile products were found in the urine of C^{14}-AAN animals, and only a minor portion of the radioactivity (2-3%) was extractable by ether from either neutral or basic urine. Apparently the 2 nitriles are metabolized differently.

Fig. 2 gives the relative C^{14} levels in body

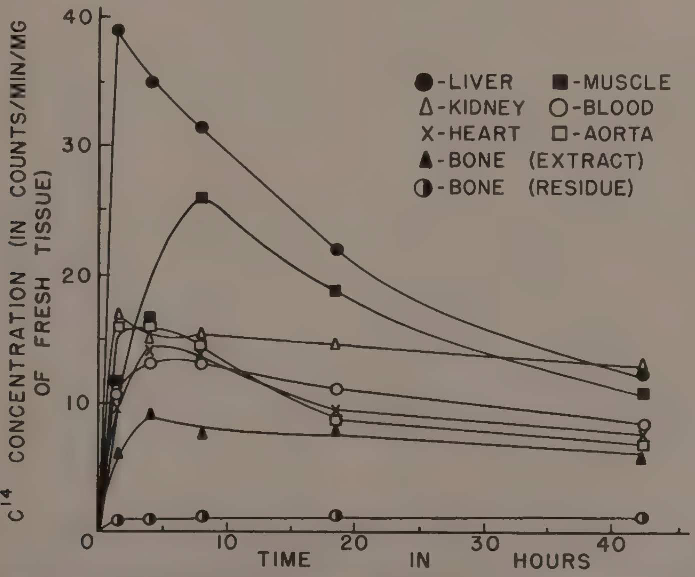

FIG. 2. Distribution of isotope in various tissues, following inj. of 10 mg of AAN-C^{14} per rat. Each point represents pooled material from 3 animals.

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§ Attempts to detect C^{14}-labeled thiocyanate by paper chromatography were unsuccessful.

TABLE II. C^{14} Distribution in Rats Fed a Stock Diet Containing 0.1% AAN-C^{14}.

Tissue	C^{14} in cts/min./g fresh tissue 6 days	10 days	μmoles of labeled substances/g tissue 6 days	10 days
Liver	7300	7600	2.25	2.34
Kidney	6500	9900	2.00	3.04
Skeletal muscle	2480	3000	.76	.93
Blood	2100	2250	.65	.69
Bone*	2280	2250	.69	.70
Epiphyseal plates of long bones	1770	1990	.55	.61

* Non-mineral extractives.

All values are for pooled tissues of 3 rats. The animals developed moderate lesions at 6 days, and typically severe lesions by 10 days.

tissues at varying times after the administration of a larger quantity of AAN-C^{14} to rats. It may be seen that liver was by far the most active tissue. After 4 hours the specific activity of muscle exceeded that of all other tissues excepting liver, and after about 30 hours, liver, muscle, and kidney were comparable in C^{14} concentration. Blood, heart, aorta, and bone (aqueous extractives) tended toward somewhat lower values, while bone residue was least active. It is evident that the radioactivity was retained by the animals to a greater degree than in Fig. 1, in which the dosage of AAN-C^{14} was smaller.

If the results in Fig. 2 are considered in terms of the total mass of each tissue, it is apparent that skeletal muscle actually accounts for a greater percentage of the isotope than does liver. Likewise, the skeleton, because of its relatively great weight and also its retention of the isotope, contained more C^{14} than any tissue except muscle, and possibly liver.

TABLE III. Isotopic AAN in Rat Tissue.

Tissue	% of C^{14} in each tissue recovered by carrier method Sacrificed 1½ hr after inj. 10 mg AAN-C^{14}	Sacrificed 18 hr after inj. 10 mg AAN-C^{14}	Fed for 10 days diet containing .1% AAN-C^{14}
Blood	73	53	27
Liver	24	16	25
Kidney	33		20
Muscle	61	43	26
Bone	90	58	31
Epiphyseal plates of long bones			39

The animals were those described in Fig. 2 and Table II.

When rats were maintained for a number of days on diets containing AAN-C^{14}, relatively high concentrations of labeled material accumulated in the body tissues (Table II). This is most evident when the radioactivities are converted into μMoles. The values for bone and epiphyseal plates, while not as high as with liver or kidney, do represent substantial accumulations of labeled carbon. Furthermore, it is noteworthy (Table III) that bone was the tissue with the highest percentage of its radioactivity in the form of free AAN; 39% of the C^{14} in the epiphyseal cartilage plates of rats on the AAN-C^{14} diet was present in the form of AAN. These plates were extremely enlarged.

TABLE IV. C^{14} in Tissue Proteins of Rats.

Tissue	Sacrificed 18 hr after inj. 10 mg AAN-C^{14}	Fed 10 days diet containing .1% isotopic AAN
Blood	10	8
Liver	13	12
Kidney		6
Muscle	4	5
Bone	6	7
Epiphyseal plates of long bones		14

The values represent % of total C^{14} in each tissue.

It is of interest that a significant part of the C^{14} in the tissues was in protein combination (Table IV). The high value for epiphyseal plates again reflects the intense synthetic activity occurring in this region. Examination of the liver protein of rats given a single injection of AAN-C^{14} disclosed that 40% of the radioactivity of the protein was due to glycine, while serine accounted for 18%. The distribution of the remaining C^{14} was not determined.

It was found that the sulfated polysaccharide isolated from epiphyseal plates was moderately radioactive (Table V). The position of the labeling was not determined. The C^{14} content of the epiphyseal cartilage protein isolated in this same experiment is given for comparison.

Indication that the C^{14} of AAN partici-

TABLE V. Labeling in Epiphyseal Plate. Rats maintained for 10 days on stock diet containing 0.1% isotopic AAN.

Substance isolated	C^{14} conc./g fresh tissue Cts/min.	 μM
Polysaccharide fraction*	95	.029
Protein	157	.048

* Approximately 10 mg were obtained per g of plates.

pated in a variety of reactions was the finding of small but definite concentrations of isotope in glycogen and lipids of liver, 4 hours after injecting AAN-C^{14} into rats. The glycogen accounted for 0.5%, and the lipids 0.6%, of the total C^{14} in this organ.

Summary. Aminoacetonitrile, with C^{14} in its cyanide group, was synthesized. When this compound was administered to weanling rats, the major portion of the radioactivity was eliminated in the urine, while a minor part appeared in respiratory CO_2. High initial C^{14} concentrations were observed in various body tissues. The proportion of this C^{14} due to unchanged nitrile decreased fairly rapidly with time. Labeling was found in tissue proteins, and to a lesser extent in liver lipids and glycogen. Also, in rats fed a diet containing isotopic aminoacetonitrile, radioactivity was detected in the sulfated polysaccharides of the enlarged epiphyseal plates of the bones. The presence of C^{14} in glycine and serine of liver protein and in urinary creatinine, allantoin, and hippuric acid, indicated an appreciable conversion of the cyanide radical into a carboxyl group. However, the major portion of the C^{14}-containing metabolites was not identified.

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1. Wawzonek, S., Ponseti, I. V., Shepard, R. S., and Wiedenmann, L. G., *Science*, 1955, v121, 63.
2. Strong, F. M., *Nutrition Revs.*, 1956, v14, 65.
3. Ponseti, I. V., Wawzonek, S., Shepard, R. S., Evans, T. C., and Stearns, G., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 366.
4. *Organic Syntheses*, 1941, Coll. v1, 298.
5. Winnick, T., *Arch. Biochem.*, 1950, v27, 65.
6. Goldsworthy, P. D., Winnick, T., and Greenberg, D. M., *J. Biol. Chem.*, 1949, v180, 341.
7. Stetten, D., and Boxer, G. E., *ibid.*, 1944, v155, 231.
8. Janes, R. G., and Winnick, T., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 226.
9. Allen, F. W., and Luck, J. M., *J. Biol. Chem.*, 1939, v82, 693.
10. Bloch, K., and Schoenheimer, R., *ibid.*, 1939, v131, 113.
11. Valentine, J. D., Gurin, S., and Wilson, D. W., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 794.
12. Shemin, D., *J. Biol. Chem.*, 1946, v162, 297.
13. Meyer, K., and Smyth, E. M., *ibid.*, 1937, v119, 507.

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Chronic Effect of Dietary Protein on Hypercholesteremia in the Rat.*

(22804)

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A diet consisting of casein, lard and dextrin was found to be atherogenic in the rat(1). Variations in fat and cholesterol content of the diet seemed to have little influence on the consequent coronary arterial lesions(2). A reduction in quality or quantity of dietary protein has raised the serum cholesterol level and promoted atherogenesis in other experimental situations(3,4).

The serum cholesterol level of adult cholesterol-fed rats rises gradually over a period of months to reach a plateau, where it remains for at least another 3 to 6 months. The serum cholesterol after 1 or 2 months may not reflect the ultimate level to be reached when the animal has finally adapted to the diet. On the other hand, the great frequency of a chronic form of pneumonitis(5), which is virtually inescapable in the rat after the age of

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* This work was made possible by grants-in-aid from Amer. Heart Assoc. and National Heart Institute of USPHS.

TABLE I. Dietary Variables Expressed as % by Weight.

Series	First				Second			Third	
Group	I	II	III	IV	V	VI	VII	VIII	IX
Casein	18.75	25	12.5	7.5	18.75	9	7.5	40	Purina lab chow
Gelatin				10.5					
Lard	30	15	15	15	30	15	15	15	
Dextrin	32.2	44	56.5	51	32.2	60	61.5	25.4	
Cholesterol	.625	1.2	1.2	1.2	1.2	1.2	1.2	1.2	

The constitution of supplementary salt mix, vitamin mix and other ingredients as well as their sources have been described previously(1,2). All groups received 0.625% choline chloride.

6 months, has led us to limit these studies to a 6-month period.

Method. Male weanling rats of the Sprague-Dawley stock were fed Purina Lab Chow until 3 months of age (250-300 g), when they were divided into dietary groups. They were fed and watered daily and weighed weekly; the food consumption was estimated daily. Each cage contained no more than 8 rats. Under seconal anesthesia and in the fasting state they were bled from the tail vein at 8 weeks, and by cardiac puncture when sacrificed at 25 weeks. Individual serum cholesterol determinations were done in duplicate by the method of Zlatkis(6) and serum phospholipid by a modification of the Fiske-SubbaRow technic(7) on aliquots of a 3:1 alcohol-ether extract of the serum. Gross autopsy of the sacrificed animals was performed, but histological examination was limited to frozen sections of the heart and liver, which were stained with Sudan III. The diets employed were variations from the original diet of Wissler, *et al.*(1), consisting of 30% lard and 18.75% casein. The level of lard was reduced to 15% in all cases, except for this original diet, and the amount of food offered was correspondingly increased. This lower fat level was introduced in order to minimize the incidence of pneumonitis and seemed to make no difference in the ultimate cholesterol level. An increase in level of cholesterol supplement from 0.31 to 0.625 or even 1.2% made no difference in the ultimate hypercholesteremia, though, as others have noted, the higher levels of dietary cholesterol accelerated the rise in serum cholesterol to that level(8). In each case, protein increments replaced dextrin, the fat level remaining constant. In 2 experiments the above pattern was followed in comparing various levels of casein from 25 to 7.5% and a combination of casein and gelatin as outlined in Table I. In a third experiment, using the diet described for group VIII, the rats were 15 months old at the onset of the experimental period, having been on a Purina Chow diet from the date of weaning and they were bled only after 6 months. In this experiment the rats in group VIII were compared with chow-fed animals of the same age. Lipoprotein concentrations of pooled serum of both groups were compared by the technic of Lewis, Green and Page(9) with the analytical ultracentrifuge.

Results. Statistical comparisons (Table II) revealed a level of serum cholesterol in animals fed 12.5% casein for 25 weeks, which was significantly lower ($P<0.01$) than in those fed 25%, 9% or 7.5%. While the cholesterol level attained with 18.75% casein is not enough higher than that reached with 12.5% to achieve statistical significance, it is significantly lower ($P<0.01$) than that resulting from 25% casein or 9% casein in the diet. While it may not be permissible to compare directly the older animals in group VIII with the other groups, their serum cholesterol was so uniquely high for rats on an *ad lib.* diet that they are included here with their chow-fed controls, group IX. No such high levels of serum cholesterol have been observed by us in many other old animals fed the 18.75% casein diet for even longer periods (1,2).

The cholesterol/lipid phosphorus ratios became generally elevated in the first and third experiments where the higher levels of protein were used, and failed to rise significantly at the lower levels of protein fed in the second

TABLE II. Comparison between Mean Serum Cholesterol and Cholesterol/Lipid Phosphorus at Various Levels of Dietary Protein.

		Diet		Wt in g			Cholesterol (mg %) ± S.E.		Cholesterol/L. phosphorus (mg %) ± S.E.	
Group	No. of rats	% casein	% gel	Initial	Final	Gain	8 wk	25 wk	8 wk	25 wk
						Exp. 1				
I	18	18.75		285	435	150	121 ± 5.0	158 ± 7.1	24.1 ± .75	25.2 ± 1.07
II	23	25		292	457	165	120 ± 3.0	224 ± 6.6	26.8 ± .70	41.9 ± 1.08
III	22	12.5		290	413	123	148 ± 6.1	148 ± 5.7	25.8 ± 1.08	41.3 ± 1.57
IV	23	7.5	10.5	295	354	59	136 ± 3.4	178 ± 6.0	23.9 ± .66	34.9 ± 2.29
						Exp. 2				
V	8	18.75		323	506	183	155 ± 3.8	161 ± 5.6	23.4 ± .4	25.9 ± 1.38
VI	8	9		302	400	88	142 ± 7.9	235 ± 9.1	23.2 ± 1.4	27.2 ± 1.40
VII	8	7.5		307	330	23	121 ± 3.0	212 ± 29.5	21.4 ± .4	24.4 ± 2.60
						Exp. 3				
VIII	10	40		510	584	74		490 ± 75.0		32.5 ± 1.90
IX	9	Chow		482	507	29		162 ± 10.2		21.2 ± .82

experiment—even though the serum cholesterol was equally elevated. In the first experiment the terminal ratios are significantly higher in groups II, III and IV than in group I ($P<0.001$), and that of group IV is significantly lower than groups II and III ($P<0.02$). The explanation for a greater disproportion between cholesterol and lipid phosphorus at 25% and 12.5% levels than is seen at 18.75% probably depends upon the fact that the percentage of lard was higher in the latter case(10). In the second experiment the phospholipid tends to rise more nearly parallel to the serum cholesterol, so that even with the higher cholesterol levels no statistically significant differences could be established in the ratios. Again in the third experiment where a very high casein level is fed and a phenomenally high serum cholesterol is seen, the cholesterol/lipid phosphorus ratio is elevated. It appears that the hypercholesteremia of high or adequate casein diets is accompanied by a dramatic increase in the cholesterol/lipid phosphorus ratio, which is not seen with the hypercholesteremia induced by the low levels.

Except for occasional pneumonitis in the older animals of the third experiment, all animals were quite healthy until sacrifice, and gave no evidence of disease on gross autopsy. Histological studies of the heart revealed no coronary artery lesions in any of the groups excepting group VIII where 3 out of 9 animals exhibited subintimal fat staining atheromatous lesions identical to those previously reported in younger rats after one year on the diet fed group I(1,2). Serum lipoprotein concentrations were examined in this group and there was a higher level of faster rising lipoprotein fractions with a lower concentration of high density lipoproteins than in the pooled sera from chow-fed controls (Table III).

Discussion. The serum cholesterol level of the rat is apparently quite sensitive to the sustained protein level in the diet. When gelatin is added to a low casein allowance, hypercholesteremia tends to be less marked (group IV compared to Group VII) though not significantly different from that seen with an equivalent amount of protein derived solely from casein (group I vs. group IV). There is no significant elevation of the cholesterol/lipid phosphorus ratio in the animals on the low casein intake, as compared with those on moderate or higher levels of casein and comparable fat intake; hence, while we may

TABLE III. mg % Lipoproteins Determined on Pooled Sera of 21-Month-old Rats Fed 40% Casein or Chow for Preceding 6 Months.

	-S 1.21*			
Diet	0–20	20–40	40–70	100–400
40% casein	95	33	65	152
	79	40	57	160
Chow	144	38	15	65
	169	43	27	96

* Flotation rate in 10^{-13} cms/sec. of macromolecules in NaCl-KBr medium of density = 1.21.

postulate an amino acid imbalance in the former case, we would anticipate a different mechanism in the latter.

Fillios and Mann(3) have shown that a hypercholesteremia develops in rats fed a methionine deficient diet, and were able partially to correct the abnormal elevation of serum cholesterol by methionine supplementation. A casein diet of 7½% is suboptimal in methionine and permits the development of a fatty liver which may be partially corrected by further protein or methionine supplement (11).

Higher levels of serum cholesterol have been observed in mice fed high casein diets (80%) than in their litter mate controls fed a low casein (10%) diet(12). Other workers have reported a reduction in hypercholesteremic response with increasing levels of dietary casein up to 60%(13). In this case, however, supplemental cholate was included in the diet, thus masking any choleretic effect of the dietary protein.

The possibility must be considered that it is decrease in carbohydrate rather than increase in protein that is responsible for the hypercholesteremia induced in groups II and VIII. The actual amount of dextrin supplied in each diet is given in Table I, and it can be seen that the percentage supplied in group VIII was almost as much as in groups I and V, so that it seems doubtful that the marked elevation in serum cholesterol in group VIII could be due primarily to the low dextrin level, even though Portman and co-workers (14) have demonstrated a difference between corn starch and simpler sugars in choleretic potential and their effect on experimental hypercholesteremia.

The group of rats fed 40% casein developed a higher cholesterol level than heretofore reported in rats on an *ad lib.* diet free of thiouracil or cholic acid supplements. This correlates with a decrease in alpha lipoprotein and an increase in lower density lipoproteins. The development of coronary atheromatous lesions required a shorter period of time than has been noted previously even in rats of the same age(1,2).

Summary. Chronic dietary experiments in rats have revealed that months are necessary to evaluate the ultimate level of serum lipids resulting from sustained feeding of various levels of dietary protein. Quantitative changes in dietary protein can influence the levels of serum cholesterol and cholesterol/lipid phosphorus ratio. Reduction or elevation of dietary casein beyond a modest range (12-18%) will lead to ultimate elevation of serum cholesterol and phospholipid under conditions herein described. The insignificant change in cholesterol/lipid phosphorus ratio at low levels of dietary casein as opposed to its elevation at higher levels suggests that different mechanisms are operating in these two situations. Unusually high levels of serum cholesterol were achieved in old rats fed 40% casein. Coronary arterial atheromatous lesions developed in one-third of these animals, but in none of the other groups in the 6-month period.

1. Wissler, R. W., Eilert, M. L., Schroeder, M. A., and Cohen, L., *Arch. Path.*, 1954, v57, 333.

2. Jones, R. J., Wissler, R. W., and Hoffman, S., *ibid.*, in press.

3. Fillios, L. C., and Mann, G. V., *Metab.*, 1954, v3, 16.

4. Li, T., and Freeman, S., *Am. J. Physiol.*, 1946, v145, 660.

5. Nelson, J. B., *J. Exp. Med.*, 1951, v94, 377.

6. Zlatkis, A., Zak, B., and Boyle, A. J., *J. Lab. and Clin. Med.*, 1953, v41, 486.

7. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem*, 1925, v66, 375.

8. Shull, K. H., Mann, G. V., Andrus, S. B., and Stone, F. J., *Am. J. Physiol.*, 1954, v176, 475.

9. Lewis, L. A., Green, A. A., and Page, I. H., *ibid.*, 1952, v171, 391.

10. Deuel, H. J., *The Lipids*, v2, 1955, Interscience Publishers.

11. Lewis, H. B., and Fajans, R. S., *J. Nutrition*, 1951, v44, 399.

12. Mayer, J., and Jones, A. K., *Am. J. Physiol.*, 1953, v175, 339.

13. Moyer, A. W., Kritchevsky, D., Logan, J. B., and Cox, H. R., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 736.

14. Portman, O. W., Lawry, E. Y., and Bruno, D., *ibid.*, 1956, v91, 321.

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Effect on Suckling Young of X-Irradiation of Lactating Mother.[*] (22805)

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X-irradiation as a general debilitating agent should affect the nursing mouse so that its suckling young would be at least deprived of some nourishment. This study was made to determine the minimum dose level of exposure of the mother which would be reflected in the growth rate of the suckling young.

Materials and method. Adult CF_1 female mice which had had previous litters were used. Only those females with litters of 8 or more were chosen, and each litter in excess of this number was reduced to 8. Two days after delivery the mothers were separated from their litters long enough to be exposed to whole body x-irradiation. The physical facilities consisted of a Quadrocondex constant potential x-ray therapy machine run at 210 KVP and 15 MA, and the filters consisted of 0.5 Al. and 0.28 Cu. The target distance to center of the body of the mouse was 50 cm and output of the machine was 97.7 r/min. in air at that distance. The exposures used ranged from 50 r to 400 r. Immediately following x-irradiation females were returned to their own litters. The simultaneous controls were not exposed to any irradiation. On the day of x-irradiation of the mother, each litter was weighed as a unit. Each litter was weighed three times each week. A total of 70 females with their litters comprised this study. For each dose level used there were at least 8 litters.

It was soon found that suckling normally continued until after day 15 when those mice which were not receiving adequate nourishment turned to the solid food provided for the mother. Therefore, the direct effect on lactation, as reflected in the growth rate, could be determined accurately only until about day 15.

Results and discussion. Data from two separate experiments are given.

Tables from 2 separate sets of data, indicate some effect on growth of suckling young even after 50 r whole body x-irradiation of the mother. There was pronounced retardation of growth where exposure of the mother was 200 r or more. The dose of 400 r, which is closer to the LD/50/30 of 625 r for this strain of mouse, did not stop lactation completely although the suckling young continued to grow at a very slow rate. Retardation of

TABLE I. Average Weights of Young Mice Nursed by X-Irradiated Mothers.

Day	Control	50 r	100 r	200 r	300 r	400 r
3	2.5	2.4	2.4	1.9	2.0	2.3
5	3.8 (.7)	3.5 (1.1)	3.2 (.8)	2.6 (.7)	2.8 (.8)	3.5 (1.2)
8	5.4 (1.6)	5.1 (1.6)	5.3 (2.1)	3.5 (.9)	3.9 (1.1)	4.4 (.9)
10	6.4 (1.0)	5.9 (.8)	6.2 (.9)	4.2 (.7)	4.8 (.9)	4.8 (.4)
12	7.2 (.8)	6.9 (1.0)	6.8 (.6)	4.8 (.6)	5.6 (.8)	4.9 (.1)
15	8.2 (1.0)	7.8 (.9)	7.6 (.8)	5.5 (.7)	5.4 (–.2)	5.1 (.2)

TABLE II.

Day	Control	50 r	100 r	200 r	300 r	400 r
3	2.1	2.0	2.5	2.0	2.1	1.9
5	3.1 (1.0)	2.9 (.9)	3.0 (.5)	3.0 (1.0)	3.0 (.9)	2.7 (.8)
8	4.4 (1.3)	4.3 (1.4)	4.2 (1.2)	4.2 (1.2)	3.9 (.9)	3.6 (.9)
10	5.5 (1.1)	5.1 (.8)	4.9 (.7)	4.8 (.6)	3.9 (.0)	4.4 (.8)
12	6.4 (.9)	5.7 (.6)	5.4 (.5)	5.6 (.8)	4.8 (.9)	5.0 (.6)
15	6.6 (.2)	6.2 (.5)	6.1 (.7)	6.4 (.8)	5.1 (.3)	5.7 (.7)

Note: Increments are in parentheses. Only one is decrement.

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[*] This work was performed under Contract AT-30-1-GEN-70 for Atomic Energy Commission.

[†] The author acknowledges with pleasure the help of Agatha Nadai.

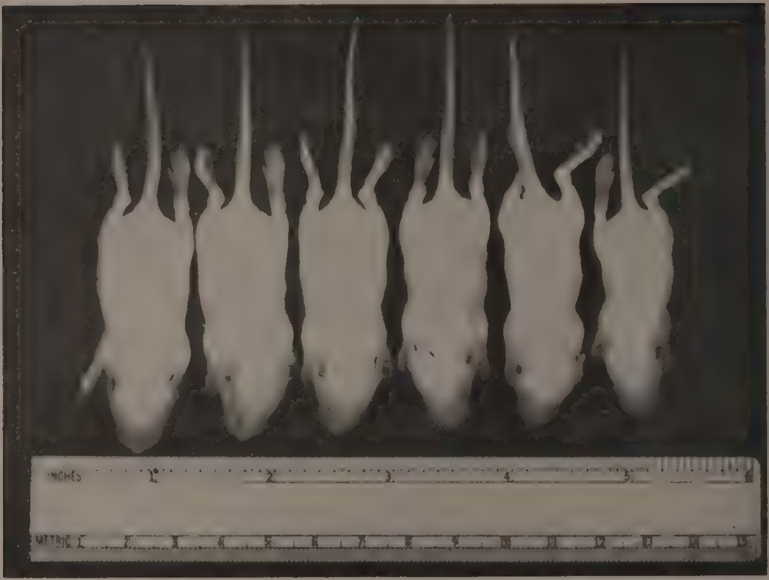

FIG. 1. Suckling mice at 10 days of age, taken from mothers variously treated on the 3rd day as follows: Left to right—Control, 50 r, 100 r, 200 r, 300 r, 400 r. Note slight stunting due to starvation, progressively greater the greater the irradiation of the mother.

growth of the suckling young appeared to be due to simple starvation and not to passage of toxic substances through the milk. This was demonstrated by replacing the irradiated mothers with normal lactating mothers of the same post-delivery age and determining the effect on the young. Invariably they regained their growth momentum almost immediately so that there could not have been any residual toxic effect. Mice which had been suckling to a 400 r mother for 15 days, when given a normal mother, approached the normal control weight by 30 days of age. True, some of these mice were able to feed on the dry food available to the mother, but the initial recovery of growth momentum was due to the abundance of normal milk.

The reciprocal exchange was made. Mice nursing to normal mothers were, at 15 days, given to previously x-irradiated (200 r or more) mothers. The growth of these mice was abruptly retarded. However, after 3 to 5 days they regained growth momentum under the supplemental influence of self-feeding on dry food. In a few cases young mice from normal mothers were given at 15 days to mothers who had been x-irradiated to 400 r and these were starved to death within 4 to 5 days.

Summary and conclusions. 1. Increasing x-irradiation of nursing female mouse caused increasing retardation of growth of suckling young. There is some effect even after 50 r exposure of the mother on the 3rd day postparturition. There was pronounced effect at 200 r or greater. 2. There is no evidence of any toxic substance passing through the milk to the young. The effect seems to be one of simple starvation, probably through general debilitation of x-irradiated mother. 3. Newborn mice starved by an x-irradiated mother will recover rapidly if given to a normal lactating mother at 15 days postparturition, and by 30 days will be essentially like controls with respect to body weight. Recovery from initial starvation appears to be complete.

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Prolactin and Thyrotrophin Content of Functional Transplantable Pituitary Tumors. (22806)

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Transplantable functional pituitary tumors described recently were classified on the basis of their hormonal secretion as indicated by changes in tumor-bearing hosts(1). Three main types have been studied extensively: adrenotrophic, thyrotrophic and mammary gland stimulating. The adrenocorticotrophin and melanophore stimulating activity of an adrenotrophic tumor(2) and the thyrotrophin (TSH) activity of some thyrotrophic tumors have been determined. The following is a study of prolactin content and a more complete account of the TSH content of various tumors.

Methods. Prolactin was assayed in pigeons by the cropgland weight increase method (3,4). Thyrotrophin was assayed in baby chicks by following the depletion of I^{131} from the thyroid(5). Each powder was a pool of tumors from several mice. All lyophilized or acetone dried tumor powders were put through a Wiley mill using a 60 mesh screen and suspensions of such powders in 0.1 M $NaHCO_3$ solution were injected using a 22 gauge needle. Heparinized blood was collected from abdominal aorta of mice. After centrifuging, the plasma was kept frozen until injected. Prolactin was extracted from 2 tumor powders using 65% ethanol at pH 9.5-10. The alkaline alcoholic extracts were adjusted to the isoelectric region, pH 5-7, and prolactin precipitated in refrigerator by addition of ethanol to a concentration of 85%. This precipitate was washed with acetone and dried. After removal of tumor, adrenals, gonads, pituitary and obvious abdominal metastases, the bodies of 9 radiothyroidectomized mice, which had had large dependent thyrotrophic tumors (Strain 77D) were frozen. Later they were ground in a Waring blendor with 3 volumes of 1% saline and then centrifuged. The cloudy liquid phase was adjusted to pH 4.7 with N/1 HCl and again centrifuged. The clear supernatant at pH 4.7, which should contain any TSH present, was adjusted to pH 7 and lyophilized for bioassay. Seven grams of powder were obtained.

Material. Tumor Strains: Thyrotrophic tumor strain 77D was induced in a C57Bl male mouse by 275 μc of I^{131}, remained dependent for 4 consecutive passages. In one line autonomy was acquired in the fifth subpassage. Thyrotrophic tumor strain 101D was induced in a C57Bl female mouse by 400 μc I^{131}, remained dependent through 2 subtransfers, but one line developed autonomy in third transfer. The present study was made with dependent tumor lines. The origin and characteristics of the mammotrophic tumor strains in mice(6) and rats(7) have been described. Characteristics of the adrenotrophic strain 2 induced by total body ionizing irradiation have been reported(1).

Results. TSH and Prolactin content of tumor powders: There is a very high potency of the 2 dependent TSH tumors (77D and 101D) (Table I). This is about 10 times that of powders from cattle or sheep pituitaries. The other tumors all gave negative tests or borderline responses at the high dose level used. These borderline tests represent concentrations of TSH that are only 1% that found in bovine pituitaries and 0.1% that found in TSH tumors.

Thyrotrophic and adrenotrophic tumors gave negative tests for prolactin. The stilbestrol dependent mammotrophic rat tumor (FR4) was uniformly positive but had only 10% concentration of prolactin found in bovine pituitaries. Powders of mouse mammotrophic tumor (OG4) gave variable results indicating that they had about 10-40% of prolactin potency of the rat tumor (FR4).

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TABLE I. Prolactin and Thyrotrophin Potency of Dried Powder of Six Transplantable Tumors.

	Transplantable pituitary tumor		TSH	Prolactin	
Species	Strain No.	Type of tumor	potency, USP U/g	Total dose, mg	Potency, IU/g
Mouse	77D	Thyrotrophic, dependent	200	200	<15
	101D	" "	100	"	"
	OG2	Adrenotrophic, autonomous	<.2	"	"
	OG3	Mammotrophic, "	"	"	"
	OG4	" "	.35	10*	10±
	"	Sample A, ♂—acetone dried	<.2	200	<15
	"	" B, ♀— " "	"	"	"
	"	" C, ♂—lyophilized	"	"	"
	"	" D, ♀— "	"	"	20
Rat	FR4	Mammotrophic, dependent	<.1	"	50
Bovine anterior pituitary gland powder			10-20	20	500

* Local cropsac assay.

TABLE II. Prolactin Potency of Crude Extracts of Mammotrophic Tumor Powders.

Tumor powder				Extract					
				Prolactin				TSH	
Species	No.	Type	Amt extracted, g	Yield, dry wt, g	Total dose, mg	Potency, IU/g	Tumor powder, IU/g*	Potency, USP U/g	Tumor powder, USP U/g*
Mouse	OG4A,B,C	Autonomous	10.5	1.2	200	44	5	<.15	<.02
Rat	FR4	Dependent	1.3	.24	20	300	56	"	<.03

* Potency of original tumor powder calculated from potency of extracts.

Crude extracts were made of pooled mammotrophic mouse tumors (OG4, samples A, B, and C) which had given negative tests for prolactin and of rat tumors (FR4) in order to test the feasibility of concentrating the activity before assaying. Both extracts tested negatively for TSH and positively for prolactin (Table II). The autonomous mouse tumor OG4 contained perhaps as little as 5 I.U./g of prolactin, or about 10% that of the dependent tumor FR4 and 1% that of bovine pituitary powder. The extract of the dependent tumor FR4 had a potency of 300 U/g which indicated a potency of 56 U/g for the original tumor powder. This confirmed the prolactin potency of 50 U/g obtained by direct assay of FR4 powder.

TSH content of tissues of the mouse: Because the blood level of TSH is high and the tumors are fairly vascular, it was thought worthwhile to determine the TSH content of various tissues of the body (Table III). Over 98% of the TSH activity was found to be in the tumor and only about 1% in the blood stream. The small amount found in the body could be due to unidentified metastases and residual blood.

Discussion. From the data in Table III it can be calculated that a 5 g tumor weighs 2,500 times the pituitary of the mouse and contains at least 5,000 times as much TSH. Also, the serum concentration of TSH is over 2,000 that of normal human blood. Only negative tests have been obtained with normal mouse serum.

TABLE III. TSH Potency of Pituitary Gland, Blood Serum, Tumor and Body of Mice with TSH Producing Tumors.

	TSH potency—USP units		
Tissues measured	Wet wt basis	Dry wt basis, U/g	Total potency, U/mouse
Tumor	40 U/g	200	200 (1)
Blood serum	1-2 U/ml	10	1-2 (2)
Body minus tumor	.1 U/g	.5	2 (3)
Pituitary	20 "	100	.04(4)

(1) Basis of a 5 g tumor. (2) Basis of 1 ml serum. (3) Basis of 20 g body wt. (4) Basis of 2 mg pituitary.

The TSH concentration in human blood serum is about 0.5 U/liter, or about 2 units per man(8). A human pituitary contains only about 5 to 10 units of TSH so that the total amount of TSH in the blood stream is as much as 20 to 40% of that in the pituitary gland. Hence, it is surprising that in mice with TSH tumors the blood content is only 1% that of its source, *i.e.*, the tumor. In other words, the pituitary content/blood content ratio in normal man is 5/1, but the tumor content/blood content ratio in mice is 100/1. The supernormal hormone concentration in the tumor tissue and blood of mice with transplantable dependent thyrotrophic pituitary tumors is most unusual and these materials may, therefore, be unusually valuable in physiological and chemical studies. It may be noted that these pituitary tumors release TSH at a site distant from the hypothalamus.

Meyer and Clifton(9) have recently shown that primary pituitary tumors induced by stilbestrol pellets in rats have the same prolactin concentration as normal rat pituitaries. These results cannot be correlated with our present data because their potency values were only relative and not in terms of International Units.

Summary. The prolactin and thyrotrophin content of various transplantable pituitary tumors was studied. 1. Prolactin was found in stilbestrol induced and stilbestrol dependent rat pituitary tumor and also in an autonomous mammotrophic mouse tumor in concentrations only 10% or less that found in bovine pituitaries. These tumors contained little or no thyrotrophin. 2. Two autonomous adrenotrophic tumors contained no detectable prolactin or thyrotrophin. 3. Two dependent thyrotrophic tumors contained no detectable prolactin while the thyrotrophin concentration was ten times that of bovine pituitaries. A five gram thyrotrophic tumor contains 5,000 times more thyrotrophin than one mouse pituitary. Blood levels of thyrotrophin were 2,000 times normal.

1. Furth, J., *Recent Progress in Hormone Research*, Academic Press, Inc., New York 1955, v11, 221.

2. Steelman, S. L., Kelly, T. L., Norgello, H., and Weber, G. F., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 392.

3. Riddle, O., and Bates, R. W., *Preparation, Assay and Actions of Lactogenic Hormone*. In Allen, E. *et al.*, (Ed.): *Sex and Internal Secretions* (2nd Ed.), Baltimore, Williams and Wilkins Co., 1939, p1088.

4. Bates, R. W., in Comroe, J. H., *Methods of Medical Research*, 1950, v2, p270.

5. Bates, R. W., and Cornfield, J. *Endocrinology*.

6. Furth, J., Gadsden, E. L., Clifton, K. H., and Anderson, E., *Cancer Research*, 1956, v16, 600.

7. Furth, J., Clifton, K. H., Gadsden, E. L., and Buffett, R. F., *ibid.*, 1956, v16, 608.

8. Gilliland, I. C., and Strudwick, J. I., *Brit. Med. J.*, 1956, v1, 378.

9. Meyer, R. K., and Clifton, K. H., *Endocrinology*, 1956, v58, 686.

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Mode of Action of Phenaglycodol, A New Neurosedative Agent.[†] (22807)

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Among a series of diols studied by Gibson, Mills and Swanson*, phenaglycodol (2-p-chlorophenyl-3-methyl-2, 3-butanediol) was outstanding because of the relatively long duration of its anticonvulsant action. While the compound was being tested for antiepileptic activity, the ward attendants reported that the children had become much easier to handle. Further clinical investigation has confirmed the impression that phenaglycodol can be classified as a tranquilizing agent of the muscle relaxant group. Since it is more potent and longer acting than some of the currently known drugs of this type further study seemed indicated.

Methods. Considerable attention has been given to careful observation of changes in behavior of unanesthetized mice, cats and monkeys given phenaglycodol. In addition, we studied the effect of the drug on selected multisynaptic reflex arcs in the cat. Flexion reflex was elicited at 10-second intervals by a 0.2 millisec. square wave 10-volt pulse to the posterior tibial nerve and recorded from the anterior tibial tendon with an isotonic lever in cats anesthetized with 50 mg/kg of chloralose. The knee jerk was obtained by tapping the patella tendon with a solenoid tapper. This monosynaptic reflex was modified by mid-brain stimulation(1,2). The biphasic pulses of an integrated square wave(3) of 1 millisec. duration at 20-50 cycles/sec. were led to a concentric bipolar electrode or to a monopolar electrode with the stereotaxic frame as an indifferent electrode. Points in the mesencephalic reticular formation were located by trial and error, which gave either facilitation or inhibition. Inhibition was induced by caudate stimulation. In the bulbar reticular formation, stimulation caused inhibition of the knee jerk and usually, apnea. In spinal cats, a comparison was made of the effect of phenaglycodol on predominantly monosynaptic inhibition of the knee jerk by ipsilateral sciatic stimulation, with the effect on a multisynaptic inhibition by contralateral stimulation of the nerve to the quadriceps muscle(1). Electrical activity of the brain was recorded in unrestrained cats from surface and deep electrodes chronically implanted. Some experiments were done on cats immobilized with suspension of dimethyltubocurarine butyrate in oil. The surface electrodes were 2-56 stainless steel machine screws that were placed in the skull to reach, but not penetrate, the dura. The deep electrodes were of 24-gauge stainless steel wire insulated to within 1 mm of the tip with Epoxylite Electrode Insulating Varnish. In chronic animals, Cannon miniature plugs or Winchester subminiature sockets were secured to the skull with dental acrylic resin. The surface electroencephalograms were picked up from bipolar connections or from one screw and an "indifferent" electrode over the frontal sinus. For the most part, the deep electrodes were recorded as a unipolar derivation with the indifferent electrode. The deep electrodes were placed in the septal region, the mesencephalic reticular formation, the ventro-posterior lateral and medial dorsal nuclei of the thalamus, the amygdala, and the caudate nucleus.

Results. Mice, after the injection of phenaglycodol in doses of 55 to 80 mg/kg intraperitoneally, show a reduction in spontaneous activity and sit quietly in the center of the observation area. Although muscle weakness can be demonstrated, the animals are usually not ataxic. With larger doses, there is a rapid loss of pinnal and righting reflex while the corneal reflex is still brisk. The onset of this action is smooth and there is little of the ataxic running about usually seen with phenobarbital.

Cats given doses of 20 mg kg intraperitoneally also became quiet. It was often

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[†] The trade-mark for Eli Lilly and Company for phenalglycodol is 'Ultran.'

* To be published.

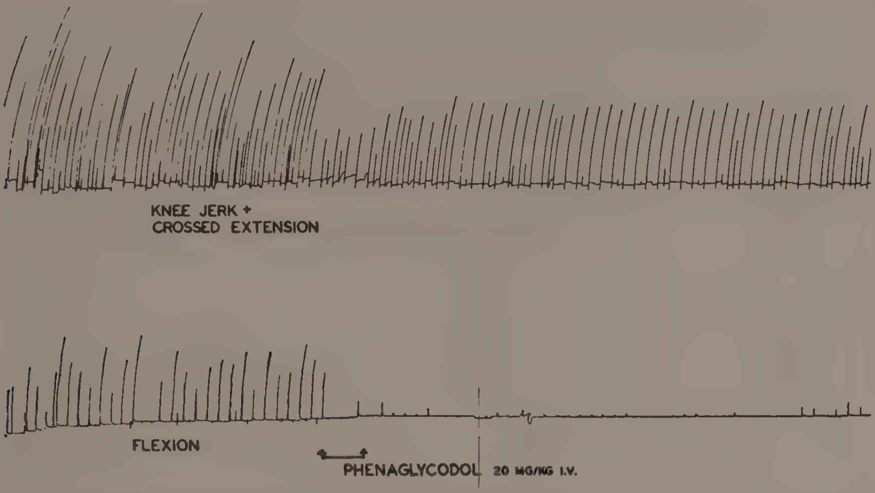

FIG. 1. Effect of phenaglycodol on spinal cord reflexes in cat. Upper trace is record of extension of the leg. Before drug, the leg extended in response to both tapping patella tendon and stimulation of contralateral posterior tibial nerve. After phenaglycodol the crossed extension disappeared while knee jerk became more consistent in amplitude.

noted that animals that were complaining about being confined in a cage seemed peaceful and content after phenaglycodol, in doses of 10 and 20 mg/kg. The animals given 40 mg/kg were ataxic and those on larger doses slept. No changes in respiration or in heart rate were noted.

Monkeys given phenaglycodol in doses of 50 to 100 mg/kg by stomach tube were less aggressive or less fearful. These animals often became playful and engrossed in their own activity. As with the other tranquilizing agents, there were a variety of patterns of behavior, depending not only on the dose of drug, but also on the character of the monkey, and on the observer and his previous experience with the colony. The larger dose caused some unsteadiness in movement. These doses were repeated daily for 6 months without appreciable change in intensity of effect. The animals usually recovered in 4 hours.

In cats anesthetized with chloralose, 20 mg/kg intravenously of phenaglycodol caused a small transient fall in blood pressure associated with some slowing of respiration. Heart rate and electrocardiogram were virtually unchanged. The pressor response to either carotid occlusion or epinephrine and the depressor response to either histamine or methacholine were unaltered. The contraction of the nictitating membrane after epinephrine or during preganglionic stimulation was not altered by phenaglycodol. Flexion reflex was usually reduced, but when the stimulus was supramaximal, there was not much change.

In experiments in which the posterior tibial nerve was stimulated, flexion reflex could be recorded ipsilaterally and crossed extension contralaterally. This stimulus was alternated with tapping of the contralateral patella tendon. Phenaglycodol in doses of 20 mg/kg intravenously caused little change in the knee jerk, but flexion and crossed extension were decreased (Fig. 1). These results were obtained in animals anesthetized with chloralose or dial-urethane solution. Further evidence of a selective depression of multisynaptic pathways in the cord was obtained from experiments in spinal cats. Phenaglycodol blocked the inhibition of the knee jerk caused by stimulation of the nerve to the contralateral quadriceps muscle but had little effect on the inhibition obtained by ipsilateral sciatic stimulation.

In experiments in which the knee jerk was

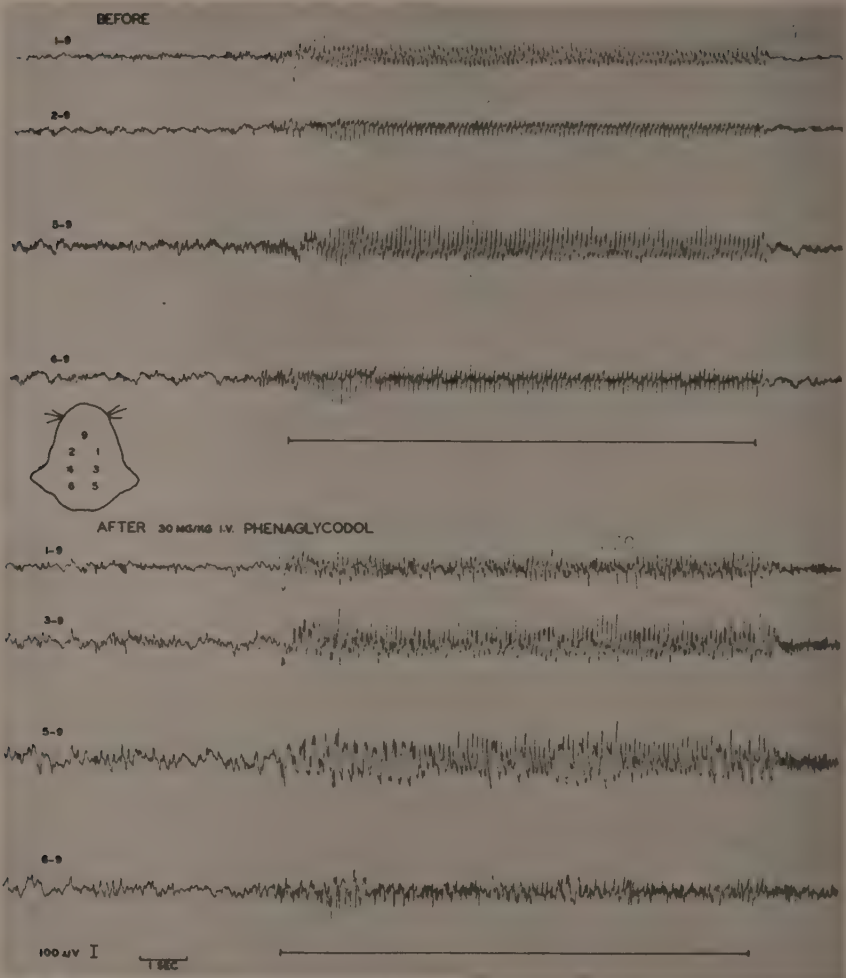

FIG. 2. Effect of phenaglycodol on EEG of cat showing change in form of recruiting response and of spontaneous record. Heavy line beneath records indicates duration of the 9 cycle/sec. stimulus.

inhibited or facilitated by supratentorial stimulation either from the mesencephalic reticular formation or from the caudate nucleus, doses of phenaglycodol of 10 to 20 mg/kg intravenously blocked the alteration of the knee jerk without changing the knee jerk itself. We did not detect a consistent difference in the effect of the drug on either facilitation or inhibition. When inhibition was obtained by stimulation of the bulbar reticular formation, however, phenaglycodol and mephenesin failed to block the change. It was characteristic of phenaglycodol that, when the drug caused a reduction in the activity of a polyneuronal arc, a simple increase in voltage of the stimulus would restore the response to or almost to the pre-drug level. The failure of phenaglycodol to block subtentorial inhibition may be related to the difference in the effect of a small increase in stimulus strength on the bulbar and mesencephalic reticular formation. In the former region a small increase in voltage changes a barely discernible decrease in knee jerk to a complete block of the reflex. In contrast, small increase of voltage of mesencephalic stimulation caused less striking changes. This difference probably reflects the smaller number of interneurons involved in mediating the effect of bulbar stimulation.

In cats immobilized with dimethyl-tubocurarine butyrate in oil, low voltage fast activity was the usual pattern of the electroencephalogram. After phenaglycodol in doses of 10 to 20 mg/kg intravenously, this desynchronized record showed a greater degree of synchrony. The voltage increased from 30-50 μV to 100-150 μV, and the frequency slowed to 9-12 c/sec. In 3 cats of this type, 9 c/sec. stimulation in the nonspecific thalamic nuclei gave a typical recruiting response in the surface leads. The threshold for this response was not changed although the form was somewhat modified after phenaglycodol (Fig. 2).

In the unrestrained cats there was a greater variety in the resting EEG pattern, but the activation pattern was usually observed. After 20 mg/kg intraperitoneally of phenaglycodol the change to a higher voltage, slower frequency record was observed. We were unable to convince ourselves that any one of the surface or deep leads could be identified as the source or starting point for the synchrony. A somewhat similar pattern of action has been obtained with pentobarbital, 10 mg/kg, but the cat seemed somewhat more sleepy after this dose. Another difference was noted in that tapping or scratching of the cage would cause an EEG arousal response after phenaglycodol, but failed to do so after pentobarbital. From these observations it would appear that phenaglycodol lies somewhere between the muscle relaxants, which depress the recruiting response but leave EEG arousal unimpaired, and the barbiturates which depress arousal and do not depress and perhaps even enhance recruiting (4).

Discussion. It seems clear from the results of these experiments that phenaglycodol is a sedative that differs materially from the barbiturates in its mode of action. The pattern of action in mice, cats and monkeys demonstrates this distinction. More detailed studies on polysynaptic pathways indicate that phenaglycodol has properties in common with the interneuronal blocking agents such as mephenesin, at least at the level of the spinal cord. The changes in the electroencephalogram demonstrate an action on higher structures, but further work will be needed to localize this effect.

Summary. 1. Phenaglycodol, a compound originally selected for clinical trial on the basis of its anticonvulsant and effective tranquilizing actions, has been shown to have a quieting effect on mice, cats and monkeys. 2. Its effects on blood pressure, respiration and electrocardiogram are minimal. 3. In studies on selected reflex arcs it has been shown to be a selective depressant of polysynaptic pathways at the spinal and supraspinal level. 4. After doses of phenaglycodol causing sedation but not sleep in cats, the electroencephalogram shows a pattern of 9-12 c/sec. synchronous activity at both surface and deep electrodes.

1. Henneman, E., Kaplan, A., and Unna, K., *J. Pharmacol. Exp. Therap.*, 1949, v97, 331.

2. Magoun, H. W., and Rhines, R., *J. Neurophysiol.*, 1946, v9, 165.

3. Lilly, J. C., Hughes, J. R., Alvord, E. C., Jr., Galkin, T. W., *Science*, 1955, v121, 468.

4. King, E. E., *J. Pharmacol. Exp. Therap.*, 1956, v116, 404.

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Fractionation of Plasma Non-Esterified Fatty Acids. (22808)

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Previous studies(1,2) have shown that non-esterified fatty acids in plasma (NEFA) fall sharply after administration of glucose or insulin, and rise after an injection of epinephrine. On the other hand, fat meals caused no significant rise of NEFA, even during the phase of hyperlipemia or in the subsequent clearing of plasma. The detailed composition of this fraction has remained undefined, except for indirect evidence that it is composed mainly of long chain fatty acids loosely coupled to albumin. Chromatographic analysis, used in the present study, shows that palmitic and oleic acids predominate in NEFA, as they do in plasma triglycerides and adipose tissue(3), and that fall in concentration of these acids accounts for the main part of the fall in concentration of NEFA after injection of insulin.

Methods. NEFA was extracted from plasma and titrated into 90% ethanol as previously described(2), then re-extracted into petrol ether (BP 50-60°C) and concentrated at 55°C under a stream of nitrogen to an acidity of 15 to 25 μeq./cc. After addition of a small amount of C^{14}-palmitic acid* to serve as a marker, the concentrate was fractionated on an octane-methyl cellosolve-water partition column(4), and alternate tubes of collected effluent were titrated for acidity or bromine uptake(5). Since palmitic and oleic acid overlap in this fractionation, a more accurate measure of their relative proportions is obtained by preliminary bromination of the extract at –20°C, thus moving the oleic acidity to a relatively empty place in the plasma spectrum. Both methods were used.

Results. Fig. 1 shows the fractionation of plasma NEFA obtained from a normal fasting subject. Fig. 2 and 3 show the changes in

TABLE I. Distribution of Fatty Acids in NEFA before and after Injection of Insulin.

Time (min.)	Plasma sample	NEFA (μEq/l)	Relative proportions (%) Stearic	Palmitic	Oleic	Not identified
−2	1	1068	6	34	54	6
0			(Insulin, 0.1 u/kg, inj. i.v.)			
45	2	527	8	36	54	2
90	3	468	10	29	42	19

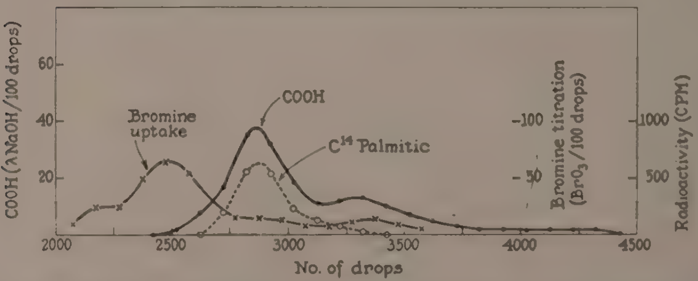

FIG. 1. Chromatographic fractionation of plasma extract. Titration of acidity (●) and uptake of bromine (×). The C^{14}-palmitic acid, added as a marker (○), contributed negligible acidity. The two partially separated peaks of acidity appear to represent palmitic and oleic acids.

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* Obtained from Tracerlab, Inc., Boston.

pattern after intravenous injection of insulin. The total NEFA and proportions of major fatty acids in one experiment are given in the table.

Summary. Palmitic and oleic acids are the predominant non-esterified fatty acids of human plasma. Their concentrations fall proportionately after an injection of insulin.

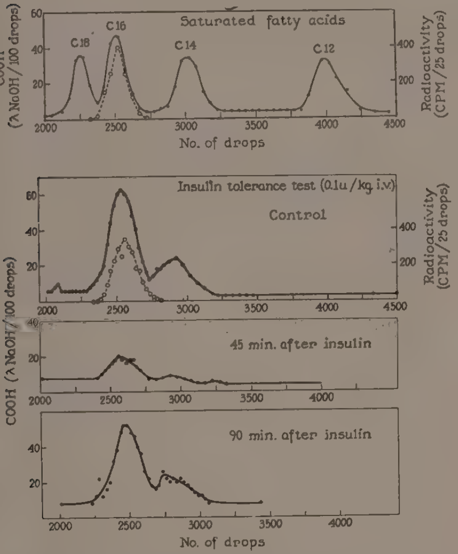

FIG. 2. Fractionation of plasma taken before and after an inj. of insulin. The upper curve shows a calibration of the column with 4 standard saturated fatty acids (solid curve) and a C^{14}-palmitic acid marker (dotted curve).

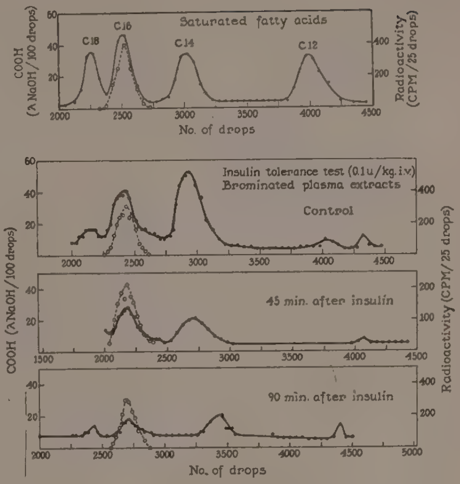

FIG. 3. Same kind of exp. as that shown in Fig. 2. The extracts were brominated before chromatography in order to increase the polarity of oleic acid and thus move the peak away from that of palmitic acid.

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1. Gordon, R. S., Jr., and Cherkes, A., *J. Clin. Invest.*, 1956, v35, 206.

2. Dole, V. P., *ibid.*, 1956, v35, 150.

3. Kelsey, F. E., and Longenecker, H. E., *J. Biol. Chem.*, 1941, v139, 727.

4. Zbinovsky, V., *Anal. Chem.*, 1955, v27, 764.

5. DuBois, H. D., and Skoog, D. A., *ibid.*, 1948, v20, 624.

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Effect of Oral Hypoglycemic Drug (Carbutamide) on Glycogen Deposition By Isolated Rat Hemidiaphragm. (22809)

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Recently sulfonylurea derivatives have been introduced in the treatment of diabetes mellitus(1-4). Although these compounds are capable of lowering the blood sugar, their mechanism of action is unknown. Originally it had been postulated that this effect was mediated through destruction of the alpha cells of the pancreas(1-3), but subsequent work has failed to confirm this(5). Mirsky *et al.* have reported an inhibition of insulinase, an enzyme system in the liver capable of degrading insulin, and concluded that such inhibition of insulin destruction was the mode of action of the sulfonylurea compounds(6). Vaughan, using a smaller amount of Carbutamide (N-p-amino-benzene-sulfonyl-N′-n-butyl urea) was unable to detect any inhibition of insulinase(7). However, in other experiments she found that Orinase (N-toluene-sulfonyl-N′-n-butyl urea) significantly reduced the effect of both epinephrine and glucagon on the liberation of free glucose from liver slices(7). The physiological significance of these findings is open to some question in view of the observation that the hyperglycemic response to epinephrine and glucagon is not altered in diabetic patients successfully treated with the sulfonylurea compounds(8).

The present studies were done to ascertain whether Carbutamide* had any insulin-like effect on the metabolism of the isolated rat hemidiaphragm or whether it would augment the effect of a known amount of insulin.

Methods. Insulin *in vitro* is known to increase the glycogen deposition by the isolated rat hemidiaphragm as compared to its control hemidiaphragm not so exposed to insulin, but otherwise handled identically(9). This increment of glycogen deposition as a result of insulin, expressed in micromoles of glucose equivalents/g of tissue, is referred to as the insulin effect. The method of determining the insulin effect was the same as reported previously(10), except for the following modifications. In the experiments designed to determine the effect of Carbutamide alone on the glycogen deposition of the diaphragm no insulin was used. Instead, one hemidiaphragm was exposed to 2 ml of 0.04 M phosphate buffer (pH 6.8) containing 2 mg of Carbutamide for 1 minute while the control hemidiaphragm was exposed to buffer alone, prior to incubation of both for 90 minutes in 0.4% glucose dissolved in buffer. At the end of the incubation, the glycogen content of each hemidiaphragm was determined. In other experiments, one hemidiaphragm was shaken with 0.2 unit of insulin in 2 cc of buffer for 1 minute while the control diaphragm was exposed to only buffer. Subsequently each hemidiaphragm was incubated for 90 minutes in 2 ml of buffer containing 2 mg of Carbutamide and 0.4% glucose. Two other series of experiments were done; in one set one-tenth (0.2 mg) the amount of Carbutamide was used, while in the other series 2 mg of sulfadiazine were substituted for an equal amount of Carbutamide. Since the usual therapeutic blood level of Carbutamide in humans is about 10 to 15 mg %, the lower concentration used represented a physiologic amount while the higher one was in the range of that used by Mirsky *et al.*(6) and Vaughan(7).

Results. Table I summarizes the results. Carbutamide by itself did not exert any insulin-like action on the glycogen deposition of the isolated rat hemidiaphragm. The effect of 0.2 unit of insulin was an increase of 6.29 micromoles of glucose equivalent per gram of tissue. When one hemidiaphragm was exposed to 0.2 unit of insulin and then incubated in glucose and buffer containing 2 mg of Carbutamide, the insulin effect was reduced to 2.90 micromoles per gram of tissue. The

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* Carbutamide used was generously supplied by Eli Lilly and Co., Indianapolis, Ind.

TABLE I. Effect of Insulin and Carbutamide on Glycogen Deposition of Rat Hemidiaphragm.

Material used with treated diaphragm	Treated diaphragm	Control diaphragm	Insulin effect
	μmoles (glucose equivalent)/g tissue		
Carbutamide, 2 mg	16.63 ± .74*	16.29 ± .90*	.34 ± .76* (6)§
Insulin, .2 unit	23.54 ± .97	17.24 ± .82	6.29 ± .59 (25)
Idem + Carbutamide, 2 mg	18.50 ± .95	15.60 ± 1.05†	2.90 ± .70 (18)
" + Carbutamide, .2 "	15.65 ± 1.71	10.74 ± 1.26†	4.91 ± .74 (9)
" + sulfadiazine, 2 "	17.37 ± 1.11	15.44 ± 1.69‡	1.93 ± 1.35 (6)

* Mean ± S.E.
† Control hemidiaphragm also treated with Carbutamide.
‡ " " " " " sulfadiazine.
§ No. of determinations in parentheses.

control hemidiaphragm was also incubated in glucose and buffer with 2 mg of Carbutamide, but it was not previously exposed to insulin. This represents a significant reduction of the insulin effect by Carbutamide ($P = < .01$). When the same experiment was repeated using a lower concentration of Carbutamide (0.2 mg), the insulin effect was 4.91 micromoles per gram of tissue, not significantly different from the insulin effect in the absence of Carbutamide. It should be noted that at this lower concentration of Carbutamide there was no enhancement of the insulin effect. If Carbutamide acts by inhibiting insulinase, and since insulinase has been reported in muscle (11), one might expect an augmentation of the insulin effect by these sulfonylurea compounds. Even though the rat hemidiaphragm is capable of a greater response to larger amounts of insulin,† the system as used in these experiments might not be sensitive enough to detect a small increment in the insulin effect. Nevertheless, when larger amounts of Carbutamide were used there was an unexpected and highly significant reduction of the insulin effect. Although these experiments do not indicate the mechanism of action of Carbutamide, they do tend to eliminate a direct insulin-like effect.

In an effort to determine the specificity of the inhibition of the insulin effect observed with the larger dose of Carbutamide, similar experiments were done using an equivalent amount of sulfadiazine. With this sulfonamide the insulin effect was also significantly reduced to 1.93 μmole/g of tissue ($P = < .01$). It thus seems that the inhibition of the insulin effect is not limited to the hypoglycemic sulfonylurea derivatives but is also shared by other sulfonamide drugs. The mechanism of this inhibition is not clear at this time. In the higher concentrations, these sulfonamide derivatives may act as non-specific protein poisons affecting several different tissue enzymes. On this basis it might also be possible to explain the inhibition of insulinase reported by Mirsky *et al.*(6) and the reduced epinephrine and glucagon response observed by Vaughan(7). The amounts of the sulfonylurea derivative used by Mirsky were 5 to 25 mg while Vaughan used 6.75 mg/100 mg of liver slices. In the present studies 2 mg were capable of inhibiting the action of insulin on the rat hemidiaphragm while one-tenth this amount had no such effect.

Summary. Carbutamide, a sulfonylurea compound capable of lowering blood sugar, did not have an insulin-like action in increasing glycogen deposition of isolated rat hemidiaphragm. Furthermore, this drug did not produce any enhancement of the insulin effect. In fact, when higher concentrations were used there was a significant inhibition of the insulin effect. This inhibition is somewhat non-specific since it was also observed when sulfadiazine was substituted for Carbutamide. It is suggested that this inhibition, the inhibition of insulinase, and the *in vitro* reduction of the epinephrine and glucagon response which have been attributed to the sulfonylurea compounds might all be due to a non-specific protein poisoning action of

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† When 2 units of insulin were used instead of 0.2 unit, the insulin effect was 10.63 ± 0.46.

these drugs in higher concentrations.

1. Franke, H., and Fuchs, J., *Deutsche Med. Wchnschr.*, 1955, v80, 1449.

2. Achelis, J. D., and Hardebeck, K., *ibid.*, 1955, v80, 1452.

3. Bertram, F., Benfeldt, H., and Otto., H., *ibid.*, 1955, v80, 1455.

4. Miller, M., and Craig, J. W., *Metabolism*, 1956, v5, 162.

5. Ferner, H., asd Runge, W., *Deutsche Med., Wchnschr.*, 1956, v81, 331.

6. Mirsky, I. A., Perisutti, G., and Diengott, D., *Metabolism*, 1956, v5, 156.

7. Vaughan, M., *Science*, 1956, v123, 885.

8. Stötter, G., Mohnike, G., Creutzfeldt, W., Sens, R., Schlagentweit, St., and Ulrich, H., *Deutsche Med. Wchnschr.*, 1956, v81, 835.

9. Gemmill, C. L., *Bull. J. Hopkins Hosp.*, 1940, v66, 232.

10. Field, J. B., and Stetten, D., Jr., *Am. J. Med.*, 1956, v21, 339.

11. Mirsky, I. A., and Broh-Kahn, R. H., *Arch. Biochem.*, 1949, v20, 1.

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Epinephrine Sensitivity of Blood Vessel Strips from Salt-Fed and Castrated Rats.* (22810)

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The observation that high sodium chloride intake increased blood pressure in rats has been made by several investigators, including Tobian, Fregly, Meneely and associates, Sapirstein, and others(1-4). The salt diet apparently induced hypertension by increasing cardiovascular reactivity, since Raab(5) found that salt and DCA potentiated the pressor effects of epinephrine in rats. That blood vessels were involved was also suggested by findings of Tobian and Binion(6) that the aorta of rats on high salt diet contained more sodium than those on low salt.

In vitro studies of reactivity of blood vessels have been made by Franklin(7) and Furchgott and Bhadrakom(8), who showed that vessel strips constricted a measurable degree, when immersed in solutions of dilute epinephrine. Since high salt diet apparently increased sensitivity of the entire cardiovascular system to epinephrine, it appeared likely that vasoconstrictor functions of blood vessels might be implicated in the response. The object of this study was to determine if blood vessels from salt-fed rats differed from those of control animals with regard to epinephrine sensitivity.

Materials and methods. In the first experiments with 50 albino rats of Sprague-Dawley strain, about same age and size (80-100 g, and 60 days old), were used. The rats were separated at random into 2 groups, one fed a normal laboratory rat diet and tap water, while the other was given the same diet except that 2% sodium chloride by dry weight had been added, and drinking water changed to 0.9% saline. At the end of 10 weeks, control and salt-fed rats were removed in pairs at daily intervals and killed by blow on the head. The aortae were removed immediately from the level of the aortic arch to the origin of renal arteries. A segment 42 mm long was cut out and split longitudinally as shown in Fig. 1A. Incomplete transverse cuts were then made alternately from each side of flattened strip as shown in Fig. 1B. Control and salt-fed preparations were suspended in aerated Evans' Ringer's solution in 37° bath. Strips were attached to long heart levers, counterbalanced so that tension supplied by weight of levers was approximately 4 g. This weight acted to elongate the cut strips in such a way that circular and spiral

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† Public Health Service Research Fellow.

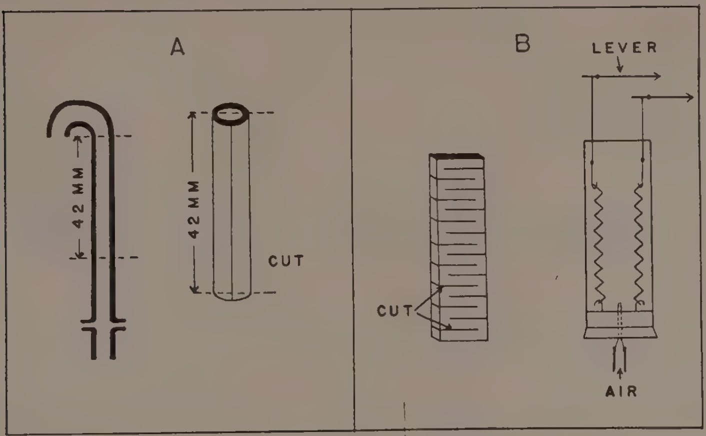

FIG. 1. Method of preparing aortic strips. A. Section cut from rat aorta and split lengthwise. B. Left, incomplete transverse cuts made across flattened strip of aorta. Right, aortic strips attached to levers and suspended in Evans' Ringer's solution.

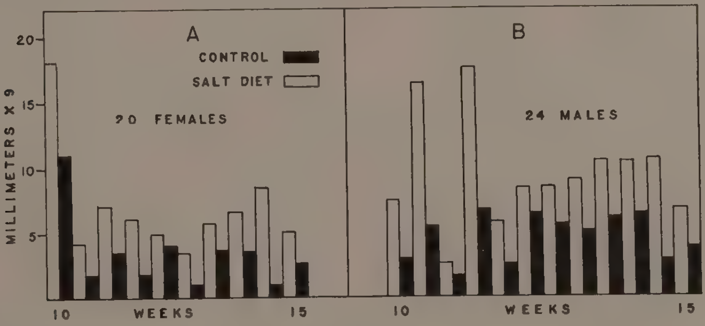

FIG. 2. Contractions produced by epinephrine in aortic strips. A. Response to epinephrine of aortic strips from control and salt-fed female rats. B. Same procedure using aortic strips from control and salt-fed male rats. Ordinate, amount of shortening of strip multiplied by magnification factor of lever system. Abscissa, number of weeks rats were kept on salt diet.

muscle layers of the aorta were oriented in a vertical direction (Fig. 1B). In preliminary experiments it was found that smooth muscle was usually contracted by the cutting procedures, and that best results were secured if the strip was allowed to relax for about 1 hour in aerated Ringer's solution previous to the testing procedures. Experimental and control strips were placed together in Ringer's solution to which 1-epinephrine[‡] was added, to make a concentration of 0.2 μg/ml. Usually 2 or 3 trials could be made for each pair of vessels provided relaxation of the muscle was induced by replacing the epinephrine solution with fresh Ringer's between tests. In a second series a similar group of 50 rats was separated as to sex, and half of the males and females castrated under ether anesthesia. The animals were allowed to recover for 3 weeks before being placed on the salt diet described above. After 10 weeks or longer the aortae of salt-fed castrated animals were compared with those of control diet castrates for sensitivity to epinephrine. As a further check on results, a group of intact rats from the same source was given either

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[‡] Supplied as Suprarenin, courtesy of Winthrop-Stearns, Inc.

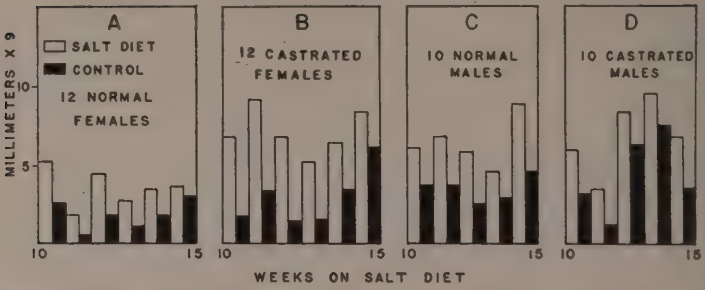

FIG. 3. Effects of castration on epinephrine contraction of aortic strips. A. Comparison of contractile response of aortic strips from control and salt-fed female rats. B. Procedures as in A, except all rats were ovariectomized previous to being placed on salt or control diets. C. Procedures as in A, except all animals were intact male rats. D. Procedures as in A, except all rats were castrated previous to being placed on salt or control diets. Ordinate and abscissa as in Fig. 2.

salt or control diets and their aortae tested as described above.

Results. Effects of salt diet on aortic epinephrine sensitivity for intact rats are shown in Fig. 2. These data indicate that responsiveness to epinephrine of aortic strips from salt-fed rats of both sexes was about double that of control strips. Statistical treatment of the data by Fisher's "t" method (9) gave a P value of 0.001, indicating a highly significant difference in sensitivity between salt-fed and control rats of both sexes. The results shown in Fig. 2 also disclosed a sex difference in the response of the aortae from salt-fed and control groups, the male aortic strips being more responsive to the same epinephrine solution than female strips. Statistical treatment gave $P = 0.001$, indicating a significant difference between the two groups.

By comparing Fig. 3A and B it can be seen that castrated females on control diet yielded aortae that were slightly but not significantly more sensitive to epinephrine than did normal females ($P = 0.2$). However, when castrated females on salt diet are compared with normal salt-fed females, (Figs. 3A and B) it is clearly evident that ovariectomy greatly increased the sensitivity of aortae to the salt diet. Statistical treatment of data gave a P value of 0.01, indicating that the difference was not likely due to chance.

In contrast to results found in operated females, the castration of males did not cause any similar increase in sensitivity of the aortae to epinephrine. When the salt diet was imposed on castrated males there was also no appreciable increase in reactivity of the aortae over those of normal males on the salt diet, as can be seen by comparing Fig. 3C and 3D. The normal rats shown in Fig. 3A and 3C confirm the finding that high salt diet increased epinephrine sensitivity of the aorta in both sexes.

Discussion. The finding that a high salt diet resulted in deposition of increased sodium in tissues, and that this was associated with development of hypertension, has been established by several investigators cited previously. From our observations on isolated blood vessel strips, it is apparent that one result of sodium deposition is an increased sensitivity of circular smooth muscle to the stimulating effects of epinephrine.

Sex differences found in the aortic response to epinephrine are in accord with the findings of Stamler(10) that salt-induced hypertension could be produced readily in male dogs and cockerels, while at most only a slight response could be produced in female dogs and laying hens. Estrogens were reported by Stamler to have anti-hypertensive effects, in that this hormone lowered blood pressure, or prevented the expected rise when DCA plus salt diets were imposed on cockerels.

Removal of ovaries and a high salt diet in our rats caused increases in aortic epinephrine response to the extent that castrated females developed about the same aortic sensitivity as males. In comparison to females, castration did not have much effect on males, since little difference was noted when the aortae of operated animals were compared to normal ones. Likewise, the salt diet did not increase aortic responsiveness to epinephrine in castrated males beyond that found in normal males. These findings indicate that the presence of female sex hormones is required to maintain a low reactivity to epinephrine in the aorta. Removal of the male hormone, on the other hand, did not change the reactivity of the aorta, nor sensitize the animal to the salt diet. The mechanism whereby the salt diet increases the reactivity of vessel strips to epinephrine may be explainable by the theory of Raab(5) that increased intracellular sodium in smooth muscle of blood vessels potentiates the constrictor effects of epinephrine, possibly by raising the membrane electrical potentials of muscle cells upon which catecholamines act.

Summary. Strips were prepared from aortae of rats and their reactivity to epinephrine tested by means of a lever system that recorded shortening of the smooth muscle. A 2% salt diet increased the sensitivity of the vessel to 1-epinephrine in both sexes. Male aortae in all instances had greater sensitivity to epinephrine than those of females under the same conditions. Castration markedly increased the sensitivity to epinephrine in females, especially when the castrates were placed on the salt diet. In contrast, male aortae were not particularly changed by castration.

1. Tobian, Louis Jr., *Am. J. Physiol.*, 1955, v181, 599.
2. Fregly, M. J., *ibid.*, 1955, v182, 139.
3. Meneely, G. R., Tucker, R. G., Darby, W. J., and Auerbach, S. H., *J. Exp. Med.*, 1953, v98, 71.
4. Sapirstein, L. A., Brandt, W. L., and Drury, D. R., Proc. Soc. Exp. Biol. and Med., 1950, v73, 82.
5. Raab, W., *Hormonal and Neurogenic Cardiovascular Disorders*, Williams and Wilkins Co., Baltimore, 1953.
6. Tobian, Louis, Jr., and Binion, John, *J. Clin. Inv.*, 1954, v33, 1407.
7. Franklin, K. J., *A Monograph on Veins*, p130-133, Charles C. Thomas, Publ., 1937.
8. Furchgott, R. F., and Bhadrakam, S., *J. Pharm. Exp. Therap.*, 1953, v108, 129.
9. Snedecor, G. W., *Statistical Methods*, Iowa State College Press, Ames, Iowa, 1946.
10. Stamler, J., *Circulation*, 1954, v10, 896.

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Insulin Secretion Following Carbutamide Injections in Normal Dogs.[*] (22811)

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The hypoglycemic action of orally active sulfonylureas may be due to one or more of the following reasons: a) destruction of the pancreatic A cells with decreased glucagon production(1), b) inhibition of one or more of the enzyme systems involved in liver glycogenolysis(2-6), c) decreased intestinal absorption of glucose(7), d) enhancement of insulin action(8), e) inhibition of the enzymes responsible for insulin degradation (9), and f) stimulation of insulin secretion (10-12). The possibility that these drugs may act through pituitary, adrenals, thyroid, parathyroid, testicles or the central nervous system appears unlikely(13) while the possibility that they may stimulate peripheral glucose utilization is uncertain(14,15). Purpose of this work was to investigate the hypothesis that carbutamide stimulates release of insulin into pancreatic blood.

Materials and methods. The problem was investigated by means of 7 pancreatic-femoral and 5 mesenteric-femoral cross-circulation ex-

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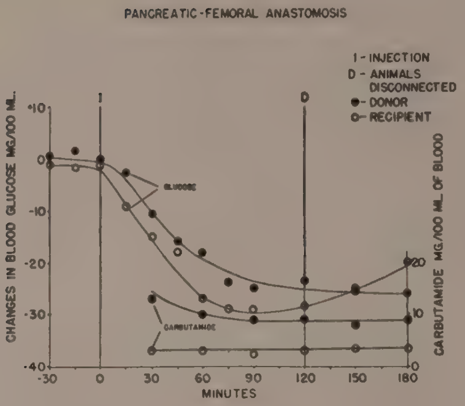

FIG. 1. Effect of carbutamide on blood glucose of dogs. Avg of 5 pancreatic-femoral cross-circulation experiments. ●, donor *dogs D*; ○, recipient *dogs R*. I = Inj. of carbutamide into *dogs D*; D = Dogs disconnected.

periments, using normal mongrel dogs of both sexes. Animals fasted for about 24 hours preceding the experiments were anesthetized with sodium pentobarbital (35 mg/kg, intraperitoneally) and heparinized.‡ The experiments were performed as described previously (16). Carbutamide was injected intravenously (50 mg/ml, 1 ml/kg, pH 8).§ Blood glucose was determined according to the method of Nelson(17), blood carbutamide according to the method of Bratton and Marshall(18). At the end of the experiment the animals were killed with excess anesthetic and portions of head, body and tail of pancreas were fixed in Bouin's solution for subsequent histological study, according to the method of Gomori(19).

Results. Results are presented in curves showing average changes in blood glucose concentration. Fig. 1 represents 5 pancreatic-femoral cross-circulation experiments in which donor *dog D* received an injection of carbutamide. During the control period preceding injection, there were no significant changes in blood sugar of either dog. Following carbutamide injection the blood sugar of dog D started to decline rapidly, reaching a minimum in about 1 hour and remaining low for the duration of the experiment. Maximum reduction varied between 25 and 60% in individual dogs D and was accompanied by similar hypoglycemia (25-68%)‖ in recipient *dogs R*. After interruption of the anastomosis, while the blood sugar of dog D remained low, that of dog R began to rise slowly toward preinjection levels. Fig. 2 represents 3 mesenteric-femoral cross-circulation experiments in which dog D received an injection of carbutamide. No significant changes in blood sugar of either dog D or Dog R were noted during the preinjection control period. Following the injection, blood glucose of dog D fell rapidly, while dog R showed a mild and not significant hyperglycemia. It should be pointed out that the maximum hypoglycemic effect of carbutamide did not occur at the same time in all animals and, therefore, the average curves are flatter than those representing individual experiments. Concentration of carbutamide in blood after initial mixing, reached values of about 8 mg % in dog D and about 2 mg % in dog R and remained essentially unchanged for the duration of the experiment. In 2 additional pancreatic-femoral and 2 additional mesenteric-femoral cross-circulation experiments, not included in the average curves, carbutamide did not cause hypoglycemia either in dog D or dog R, al-

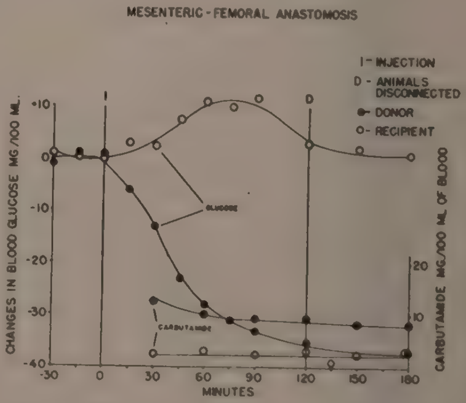

FIG. 2. Effect of carbutamide on blood glucose of dogs. Avg of 3 mesenteric-femoral cross-circulation experiments. Symbols as in Fig. 1.

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‡ Heparin was gift of Dr. Stanley Hier of Wilson Laboratories.

§ Carbutamide was gift of Dr. W. R. Kirtley of Lilly Research Laboratories.

‖ $P < 0.01$.

though the same dose had been used in all experiments and the same blood concentrations had been obtained. No significant changes were noted in the general appearance, in granules and other staining characteristics of A and B cells of the islets of Langerhans.

Discussion. The results suggest that carbutamide hypoglycemia in dog D is accompanied by a release of insulin into the blood of the pancreatic vein and that this insulin is carried through the pancreatic-femoral anastomosis, causing hypoglycemia in dog R. Small amounts of carbutamide are carried through the anastomosis also, but carbutamide concentration in blood of dog R appears to be too low to cause significant changes in blood sugar. This is shown by the fact that when dog R receives mesenteric instead of pancreatic blood from dog D, no hypoglycemia occurs, even though concentration of carbutamide in blood of dog R is approximately the same in both types of experiment. Failure of blood sugar of dog R to decrease in those experiments in which dog D did not respond to carbutamide, the slow return toward normal of blood sugar in dog R after separation from its extra insulin supply and the sustained hypoglycemia in dog D under the lasting effect of the injected drug, lend further support to the stated hypothesis. These results are in agreement with those obtained by Loubatieres using p-amino-benzene-sulfamido-isopropyl thiodiazole(10) and with the fact that carbutamide has no hypoglycemic effect in the absence of pancreatic tissue(10) and in those diabetic patients most likely to have little or no insulin in their pancreas(20). The available data do not permit the differentiation between the release of preformed insulin and true insulin secretion, although the fact that the B cells did not appear degranulated suggests active secretion. This is suggested also by the observation that carbutamide stimulates the growth of islet tissue in normal(12) and alloxan-diabetic animals(10).

Summary and conclusions. The mechanism of the hypoglycemic action of carbutamide in normal dogs was studied by means of pancreatic-femoral and mesenteric-femoral cross-circulation experiments. The results of these acute experiments suggest that: 1) carbutamide hypoglycemia is accompanied by secretion of insulin; 2) carbutamide does not cause degranulation or other significant changes in appearance of pancreatic A and B cells; 3) clearance of carbutamide from blood is relatively slow, as reported by others(21); 4) some dogs are refractory to the hypoglycemic action of carbutamide in doses of 50 mg/kg I.V. The results are consistent with the hypothesis that insulin secretion is one of the results of carbutamide action. The continued administration of carbutamide in animals and men with reduced pancreatic reserve may stimulate the islets of Langerhans to regeneration or to exhaustion. Although some evidence suggests that islet growth does occur (10,12), the second alternative has not been ruled out and should be investigated before the sulfonylureas can be recommended for the prolonged treatment of diabetes mellitus.

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1. Holt, v.C., Holt, v.L., Kroner, B., and Kühnau, J., *Arch. exp. Path. u. Pharmakol.*, 1955, v224, 66.

2. Anderson, G. E., Perfetto, A. J., Termine, C. M., and Monaco, R. R., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 340.

3. Clarke, D. W., Davidson, M., Schönbaum, E., and Senman, H., *Canadian Med. Assn. J.*, 1956, v74, 966.

4. Hawkins, R. D., Ashworth, M. A., and Haist, R. E., *ibid.*, 1956, v74, 972.

5. Vaughan, M., *Science*, 1956, v123, 885.

6. Tybergheim, J. M., Halsey, Y. D., and Williams, R. H., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 322.

7. Friedlich, T. L., Ashworth, M. A., Hawkins, R. D., and Haist, R. E., *Canadian Med. Assn. J.*, 1956, v74, 973.

8. Garattini, S., and Tessari, L., *Clin. Terap.*, 1956, v10, 418.

9. Mirsky, I. A., Perisutti, G., and Diengott, D., *Metabolism*, 1956, v5, 156.

10. Loubatières, A., *Presse Médicale*, 1955, v63, 1701, 1728.

11. Kracht, J., and Rausch-Stroomann, J. G., *Naturwiss.*, 1956, v43, 180.

12. Ashworth, M. A., and Haist, R. E., *Canadian Med. Assn. J.*, 1956, v74, 975.

13. Loubatières, A., Bouyard, P., Fruteau de Laclos, C., and Sassine, A., *Comptes Rendue Acad. Sci.*, 1956, v242, 2044.

14. McKenzie, J. M., Marshall, P. B., Stowers,

J. M., and Hunter, R. B., *British Med. J.*, 1956, v1, 448.

15. Canal, N., Garattini, S., and Tessari, L., *Boll. Soc. it. Biol. sper.*, 1956, v32, 1.

16. Foà, P. P., Weinstein, H. R., and Smith, J. A., *Am. J. Physiol.*, 1949, v157, 197.

17. Nelson, N., *J. Biol. Chem.*, 1944, v153, 375.

18. Bratton, A. C., and Marshall, E. K., Jr., *ibid*, 1939, v128, 537.

19. Gomori, G., In Ferner, H., *Das Inselsystem des Pankreas*. Stuttgart: G. Thieme, 1952.

20. Wrenshall, G. A., and Best, C. H., *Canadian Med. Assn. J.*, 1956, v74, 968.

21. Franke, H., and Fuchs, J., *Deut. med. Wschr.*, 1955, v80, 1449.

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Plasma Phospholipide Synthesis in the Eviscerated Rabbit.* (22812)

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Fishler *et al.*(1) studied the synthesis of plasma phospholipides in fasting hepatectomized dogs with P^{32} and Goldman *et al.*(2) repeated this study with C^{14}-labelled palmitic acid. From this work it was concluded that the liver is the major source of plasma phospholipides in the fasting dog. Plasma phospholipides of rats in the post-absorptive state were also found to originate in the liver(3). On the other hand, Ranney *et al.*(4) demonstrated that, in the chicken, extrahepatic tissues contribute phospholipides to the plasma.

The following experiments were conducted to determine the site(s) of plasma phospholipide synthesis in the rabbit.

Methods. Normal white rabbits maintained on a diet of 100 g Purina rabbit chow per day, and fasted 24 hours prior to the experimental period, were sedated with intraperitoneally administered urethane (750 mg/kg) and anesthetized 30 minutes later by intravenously administered sodium pentobarbital (15 mg/kg). The gastrointestinal tract and spleen, and in some instances the kidneys, were removed through a median abdominal incision. The liver was left *in situ* isolated from the hepatic artery and portal vein. Before closing the incision, bandages wet with warm saline were placed in the abdominal cavity. Eviscerations were generally completed within 10 to 20 minutes, following which the rabbits were kept warm by application of external heat. Immediately after evisceration, rabbits received an intravenous dose of 100 mg glucose (10% in saline), followed by 100 mg/kg at 30 minute intervals through the remainder of the experiment(5). Control animals were sham operated, some receiving glucose, others not. Rabbits were injected intravenously with 0.5 to 1.0 mc of labelled phosphate and sacrificed four hours later by intravenous administration of sodium pentobarbital. Two series of experiments were performed. In series I plasma and tissues were extracted with alcohol-ether, petroleum ether and analyzed as previously described

TABLE I. Tissue Phospholipide Concentrations.

Tissue	Sham operated		Eviscerated	
	mg P/g			
Series I*				
Liver	1.36‖	± .02§	1.11‖	± .02
Kidney	1.11	± .03	1.12	± .01
Muscle	.283	± .010	.272	± .009
Lung	1.13	± .01	1.11	± .05
Plasma (4 hr)	.0359	± .0065	.0273	± .0050
Series II†				
Liver	1.20‖	± .06	.995‖	± .012
Kidney	1.02	± .07	1.03	± .11
Muscle	.310	± .022	.311	± .24
Lung	.990	± .008	.944	± .033
Plasma‡ (0 hr)	.0416	± .0052	.0494	± .0156
(4 hr)	.0481‖	± .0076	.0332‖	± .0089

* 5 sham-operated, 6 eviscerated rabbits.
† 4 " , 4 " " .
‡ 9 " , 9 " " .

§ Stand. error $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

‖ Diff. significant at 5% level.

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* Supported by grant H-1238 from National Heart Institute, N.I.H., U.S.P.H.S.

TABLE II. Specific Activity[*] of Tissue Phospholipides.

Tissue	Sham operated	Eviscerated
Series I[†]		
Liver	14.9[‖] ± 2.1[¶]	.104[‖] ± .02[¶]
Kidney	33.2[‖] ± 3.7	58.1[‖] ± 2.8
Muscle	.480 ± .063	.746 ± .121
Lung	8.07 ± 1.00	13.8 ± 1.0
Plasma	3.21[‖] ± .65	1.22[‖] ± .28
Series II[‡]		
Liver	12.9[‖] ± 1.6	.120[‖] ± .049
Kidney	29.8 ± 2.2	44.6 ± 5.7
Muscle	.707 ± .159	1.34 ± .28
Lung	9.97 ± 1.16	11.3 ± 2.0
Plasma[§]	3.36[‖] ± .36	1.29[‖] ± .26

[*] Specific activity $= \frac{\% \text{ injected } P^{32}}{\text{g lipide phosphorus}}$.

[†] 5 sham-operated, 6 eviscerated rabbits.

[‡] 4 ” , 4 ” ” .

[§] 9 ” , 9 ” ” .

[‖] Diff. significant at 5% level.

[¶] Stand. error.

(6,7). In series II, experimental procedures were modified as follows: a) zero time ($t = 0$) blood samples were taken for phospholipide analysis; b) plasma acid soluble specific activities were determined on trichloroacetic acid supernatants 2 and 4 hours after administration of P^{32}; c) plasma and tissues were extracted by the chloroform-methanol procedure of Folch *et al.*(8). Tables I and II as well as further unpublished observations indicate that the two extraction methods gave comparable results for specific activities even where the concentrations differed somewhat.

Results. No significant differences in concentration of lipide phosphorus of kidney, muscle, and lung were observed between normal and eviscerated animals (Table I). Only liver showed a decrease of 17-18% in the eviscerates. Although there was no difference in plasma lipide phosphorus concentration between sham operated and eviscerated animals of series I, inspection of series II would appear to show a decrease in the eviscerates. However, the more critical comparison of zero time and 4 hour plasma phospholipide levels in each eviscerated rabbit failed to show a significant drop in concentration.

Table II summarizes the specific activity data. In control rabbits, the specific activity of liver, kidney and lung exceeded the specific activity of plasma phospholipides. Muscle, however, showed a specific activity less than that of plasma excluding this tissue as a likely source of plasma phospholipides. In eviscerated rabbits liver specific activities were 7 to 9% of the control values, evidence that liver circulation was almost completely absent. The mean plasma phospholipide specific activities in series I and II were 8-12 times as great as those observed in liver. It is apparent that labelled phospholipides from sources other than liver found their way into plasma. Since the specific activities of kidney and lung phospholipides exceeded those of plasma phospholipides, these tissues could contribute phospholipides to the plasma pool.

In an attempt to localize further the possible sites of plasma phospholipide synthesis 9 rabbits were eviscerated: in 5 animals the kidneys were left in (eviscerated controls) and in 4 they were removed (eviscerated nephrectomized). Specific activities of plasma and liver phospholipides in eviscerated controls were 1.07 ± 0.11 and 0.071 ± 0.028, respectively, which agree with the animals of Table II. In the nephrectomized animals, however, the plasma phospholipide specific activity had decreased to 0.462 ± 0.016. It is thus evident that plasma phospholipide specific activity is reduced to ⅓ of normal upon removal of liver, intestine and spleen and that by removal of kidneys it is further reduced to about 1/10th of normal. That the small amount of labelled phospholipide appearing in plasma of eviscerated nephrectomized animals is not derived from liver is shown by the fact that the specific activity of plasma phospholipide of individual animals was 10-100 times that of liver.

In series I, kidney phospholipides showed a significantly higher specific activity in eviscerates than in controls. In order to explain this difference and the similar tendency towards higher specific activity in muscle and lung, ratios of tissue phospholipide specific activity to 4 hour plasma acid soluble phosphate specific activity were determined in series II. These ratios represent an index of the rate of synthesis of phospholipide from their phosphate precursors. No evidence of altered tissue phospholipide synthesis was found in eviscerated rabbits. It appears, therefore, that the tendency towards higher specific activity in eviscerated groups is related to decrease in total phosphate pool and a subsequent increase in specific activity of phospholipide precursors.

Summary. In the rabbit, plasma phospholipide synthesis continued at a reduced rate in absence of liver, intestine and spleen. Removal of kidney further reduced plasma phospholipide synthesis. The rate of synthesis of kidney, lung or muscle phospholipides from phosphate precursors does not appear to be altered by evisceration.

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1. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, 1943, v150, 47.

2. Goldman, D. S., Chaikoff, I. L., Reinhardt, W. O., Entenman, C., and Dauben, W. G., *ibid.*, 1950, v184, 727.

3. Zilversmit, D. B., Bollman, J. L., *Arch. Biochem. Biophys.*, 1956, v63, 64.

4. Ranney, R. E., Chaikoff, I. L., and Entenman, C., *Am. J. Physiol.*, 1951, v165, 596.

5. Bergman, H. C., and Drury, D. R., PROC. SOC. EXP. BIOL. AND MED., 1937, v37, 414.

6. Zilversmit, D. B., Shore, M. L., Ackerman, R. F., *Circulation*, 1954, v9, 581.

7. King, E. J., *Biochem. J.*, 1932, v26, 292.

8. Folch, J., Ascoli, I., Lees, M., Meath, J. A., LeBaron, F. N., *J. Biol. Chem.*, 1951, v191, 833.

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Metabolic Conversion of Primidone (Mysoline) to Phenobarbital.* (22813)

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5-Ethyldihydro-5-phenyl-4,6-(1*H*,5*H*)-pyrimidinedione (primidone, Mysoline), a drug used in the treatment of epilepsy, differs structurally from phenobarbital only in that the former has a methylene group in the 2-position and the latter a carbonyl group. The only metabolic reaction that primidone has heretofore been reported to undergo is conversion to 2-ethyl-2-phenyl malonamide(1). Another reaction that suggested itself as a possibility is oxidation of the methylene group in primidone to yield phenobarbital as a product. The present investigation has shown that this reaction does indeed occur, and this report concerns a study of this metabolic conversion and its significance in therapeutics.

Methods. Administration of primidone. Crystalline primidone supplied through the kindness of Dr. A. Stanley Cook, Ayerst Laboratories, N. Y. City, was used in experiments on dogs. It was administered in gelatin capsules by mouth. The patients received commercial tablets. *Lack of contamination of primidone with phenobarbital.* A sample of crystalline primidone was partitioned between ether and water in a countercurrent design calculated to effect 99% separation of primidone and phenobarbital. Spectrophotometric examination of the fractions that would have contained phenobarbital demonstrated that the sample of primidone could have contained no more than 0.01% of phenobarbital. Information obtained from the manufacturer indicates that the method of synthesis excludes the possibility of any initial contamination with phenobarbital. *Spectrophotometric measurements* were made with a Beckman DU ultraviolet spectrophotometer. *Isolation of phenobarbital and p-hydroxyphenobarbital from urine.* The procedure described by Butler(2) was used. All samples of urine were subjected to acid hydrolysis. *Determination of phenobarbital in plasma.* For dog plasma the method of Butler *et al.*(3) was used. Unchanged primidone does not interfere to a significant extent with this method. A con-

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† Public Health Service Research Fellow of N. I. Neurological Diseases and Blindness.

centration of 500 mg/l of primidone is measured as an apparent phenobarbital concentration of 2 mg/l. Plasma from patients receiving primidone and diphenylhydantoin was subjected to a countercurrent distribution procedure for the purposes of separating phenobarbital from diphenylhydantoin and of furnishing evidence of the identity of the phenobarbital in plasma. Samples of 10 to 15 ml of plasma were extracted with ether, the ether evaporated, and residues put through a 30 cell extraction train of the type described by Craig *et al.*(4). The light solvent was a mixture of 30% (v/v) ethyl ether and 70% 2,2,4-trimethylpentane and the heavy solvent a buffer consisting of 0.75 mole $K_2HPO_4 + 0.25$ mole KH_2PO_4 per 1 (pH 7.2). Each effluent fraction of light solvent was extracted with 4 ml of a buffer of pH 11 (0.10 mole $NaHCO_3 + 0.09$ mole NaOH per l). Concentration of phenobarbital was calculated from the difference between absorbancy of the buffer extract at 240 mμ and that at 260 mμ. With this system of solvents phenobarbital appears in maximal concentration in the nineteenth effluent fraction of light solvent. From the amount of phenobarbital in the peak fraction the concentration in original sample of plasma was calculated.

Results. Phenobarbital and p-hydroxyphenobarbital in urine. Primidone was administered to a dog in a daily dose of 200 mg/kg for 6 days. Urine was collected for 7 days beginning the day after first dose. From this urine there were isolated in crystalline form 77 mg of phenobarbital and 16 mg of *p*-hydroxyphenobarbital. These products were identified by melting points and mixed melting points. From a 21 hour specimen of urine from a patient receiving a daily dose of 1 g of primidone there was isolated in crystalline form 2.5 mg of *p*-hydroxyphenobarbital, which was identified by its absorption spectra in media of different values of pH. Phenobarbital was not isolated in crystalline form, but in the fraction in which it would be expected there was a substance with absorption spectra characteristic of phenobarbital. The amount of phenobarbital as calculated from absorption measurements was 0.9 mg.

Phenobarbital in plasma. By spectrophotometric procedures the presence of phenobarbital has been demonstrated and its concentration measured in samples of plasma from 2 dogs and 2 patients receiving primidone. The dogs were given, respectively, 200 and 250 mg/kg of primidone daily for 6 days. The patients were a 79 kg man receiving 1 g of primidone plus 0.4 g of diphenylhydantoin daily and an 81 kg man receiving 2 g of primidone plus 0.3 g of diphenylhydantoin daily. The former patient had been receiving primidone for 3 weeks and the latter for 8 weeks. Both denied taking any drugs other than those prescribed, *viz.* primidone and diphenylhydantoin.

The methods used for measuring phenobarbital both in dog plasma and in human plasma are not subject to interference from unchanged primidone. In samples of plasma from both species it was shown that the substance determined as phenobarbital has spectra characteristic of that compound both in the univalent anionic form and the bivalent anionic form. When extracts of human plasma were passed through the Craig countercurrent distribution train, the material determined as phenobarbital appeared in maximal amount in the nineteenth effluent fraction, the same fraction in which phenobarbital is in maximal amount. From the tenth to the thirtieth fraction, the amount of the material determined as phenobarbital conformed closely to that expected for pure phenobarbital. Thus in the fractions in this range the material determined as phenobarbital behaves as a homogeneous substance with a partition coefficient between the organic solvent and the buffer identical to that of phenobarbital. The evidence seems conclusive that phenobarbital is present in plasma after the administration of primidone and that the analytical methods used have not measured a significant amount of any substance other than phenobarbital.

In the dogs receiving primidone for 6 days there was progressive accumulation of phenobarbital in the plasma, the highest concentration being reached on the day after dosage

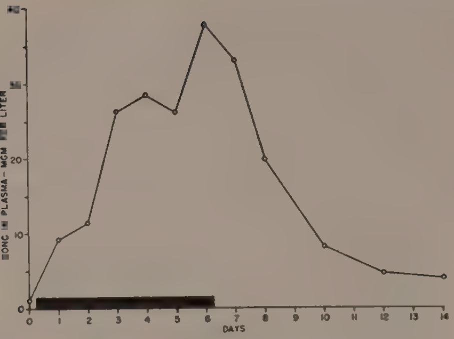

FIG. 1. Plasma phenobarbital concentrations resulting from oral administration of 200 mg/kg of primidone daily for 6 days to a 9.7 kg female dog. Period of drug administration is indicated by the bar.

was discontinued. The peak concentration was 27 mg/l in the dog on a daily dose of 250 mg/kg and 38 mg/l in the dog on a daily dose of 200 mg/kg. Fig. 1 shows the course of accumulation and elimination of phenobarbital in the latter dog.

In the patient receiving 1.0 g of primidone daily the plasma concentration of phenobarbital was 16 mg/l. In the patient receiving a daily dose of 2.0 g the plasma concentration of phenobarbital was 39 mg/l.

Discussion. The isolation of phenobarbital and its hydroxy derivative from urine and the demonstration of a substance in plasma with distribution coefficient and absorption spectra characteristic of phenobarbital give unequivocal evidence that primidone is in part converted to phenobarbital. During the course of this investigation it came to our attention that G. L. Plaa, J. M. Fujimoto, and C. H. Hine at the University of California, San Francisco, had independently found evidence of the presence of a substance with the spectral characteristics of phenobarbital in the blood of patients and in the urine of rats receiving primidone.

Comparison of the plasma concentrations of phenobarbital resulting from the administration of primidone and of phenobarbital itself permits a rough estimate of the proportion of primidone converted to phenobarbital. Calculations based on the phenobarbital concentrations found in other dogs and other patients receiving phenobarbital in chronic schedules lead to the conclusion that the dogs of the present study converted of the order of 5% and the patients of the order of 15% of the administered dose of primidone to phenobarbital. Thus the extent of the conversion is only small; but owing to the high dosage in which primidone is used and the slow rate at which phenobarbital is eliminated, that product may accumulate to reach levels sufficient to exert a significant effect. The concentrations of phenobarbital found in the plasma of the two patients of this study are in the range of those found in patients treated with antiepileptic doses of phenobarbital itself. There can be no doubt that concentrations of phenobarbital as high as those present in these two patients can be effective in suppressing seizures even in the absence of other drugs. While it seems clear that phenobarbital must, in some patients at least, make a significant contribution to the therapeutic effects of primidone, the role of the unchanged drug is not clear. If, as has been reported, seizures can be controlled in some patients by primidone and not by phenobarbital alone, the therapeutic results in such patients cannot be attributed entirely to the phenobarbital produced by the oxidation of primidone.

Summary. Primidone (Mysoline) is in part converted to phenobarbital by dog and by man. Concentrations of phenobarbital found in plasma of patients receiving therapeutic doses of primidone are high enough to exert a significant antiepileptic effect.

We are indebted to Dorothy Johnson for technical assistance and to Dr. Thomas W. Farmer for making patients available for some of these studies.

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1. Bogue, J. Y., and Carrington, H. C., Personal communication cited by Goodman, L. S., Swinyard, E A., Brown, W. C., Schiffman, D. O., Grewal, M. S., and Bliss, E. L., *J. Pharmacol. and Exp. Therap.*, 1953, v108, 428.

2. Butler, T. C., *J. Pharmacol. and Exp. Therap.*, 1956, v116, 326.

3. Butler, T. C., Mahaffee, C., and Waddell, W. J., *ibid.*, 1954, v111, 425.

4. Craig, L. C., Hausmann, W., Ahrens, E. H., Jr., and Harfenist, E. J., *Anal. Chem.*, 1951, v23, 1236.

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Reaction of Viral Hepatitis Sera with *M. rhesus* Erythrocytes.* (22814)

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Latent or asymptomatic hepatic disease has been demonstrated in apparently healthy blood donors, and has been shown to be transmissible(1). In a recent study(2) 3655 apparently healthy blood donors were given a series of hepatic tests. According to the authors' evaluation 29% of these showed abnormalities, 17% "moderate" and 12% "severe." The authors concluded there was evidence that some, but not all, asymptomatic carriers will be excluded by the "hepatic test" screen, but that elimination of so large a fraction of blood donors would create a serious problem in supplying donor blood. It is generally agreed that some type of screening is needed, and that absence of jaundice during a number of months preceding blood donation does not insure freedom from infectivity.

Heterospecific serological tests have been found very helpful in the laboratory study of infectious diseases; agglutination of sheep erythrocytes in serum from patients with infectious mononucleosis has become a definitive test in diagnosing this condition. This suggested the possibility that a similar test might be found which would aid in the detection of viral hepatitis. Accordingly, sera from individuals diagnosed as having viral hepatitis were tested for their ability to agglutinate red cells of mouse, sheep, dog, guinea pig, steer, chicken, rabbit, horse, and So. American ring-tailed monkey, with no evidence that the cells could yield useful information. However, when red cells of rhesus monkey were used, agglutination was observed to a degree which was noticeably greater than was the sera from random controls. Erythrocytes from this species were therefore chosen for more intensive study.

Methods. Macacus rhesus red blood cells were collected in A. C. D. solution and stored in the refrigerator. A working suspension was prepared by washing the cells 3 times in 0.9% NaCl solution, resuspending them after the last washing to make up a 2% suspension by volume. The working suspension is made up just before using, and unused portions are discarded. The cells stored in A. C. D. solution gradually lose their sensitivity and should not be used after 10 days' storage. Sera to be tested were inactivated at 56C° for 30 minutes. 0.2 ml of serum was added to 0.2 ml of 0.9% NaCl solution and mixed; 0.2 ml of this dilution was transferred serially through 6 tubes, each containing 0.2 ml of diluent, giving a series of dilutions ranging from 1:2 to 1:64. 0.2 ml of cell suspension was added to each tube and the set was incubated for 1 hour at 37°C. Cells were sedimented by slow centrifugation (1000 r.p.m. for 2 min.) and degree of agglutination was estimated after gentle resuspension. The end-point was considered to be the tube containing the highest dilution of serum in which a definite agglutination was macroscopically visible. One of the problems in this kind of study is selection of a control group which may be presumed to be free from false positive reactions. In this case we chose sera from the pilot tubes of bank blood, since donors are questioned as to possible attacks of jaundice in the past, and must conform to certain physical standards indicating their apparent healthy condition. Even so, we must assume that some of these may be asymptomatic carriers of one of the hepatitis viruses, so the occurrence of positive reactions in this group cannot be considered as being false positives. In such a situation where control and experimental groups may overlap it is necessary to rely upon the differences in distribution of the results rather than upon absence of any positive reactions in the control group.

In addition to the control group, 3 others were studied. These included a group of patients diagnosed as having viral hepatitis,

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* Appreciation is expressed for the assistance of Lucille Raitch, and Monica Stevens.

TABLE I. Agglutination Titers against Rhesus Erythrocytes.

Group	Highest serum dilution giving a positive test Under 1:2	1:2	1:4	1:8	1:16	1:32	Total
I. Blood donors	65	18	27	12	3	0	125
II. Jaundiced, exclusive of viral hepatitis	8	4	2	0	0	0	14
III. Infectious mononucleosis	2	5	1	4	3	0	15
IV. Viral hepatitis	0	2	3	8	5	1	19

another group of jaundiced patients whose liver pathology was due to extrahepatic obstruction, Laennec's cirrhosis or malignancy, and a third group of individuals whose illness was diagnosed as infectious mononucleosis.

Results. The results of the agglutination tests are listed in Table I and presented graphically in Fig. 1. Table I shows that distribution of the titers in the second group (patients whose icterus was not due to viral hepatitis) did not differ significantly from the blood donor group. Therefore these two were combined in Fig. 1 to form the control group. Sera in this group were found to have comparatively low titers, 124 of the 139 specimens (89%) being less than 1:8. On the other hand, sera of the viral hepatitis group showed higher titers, 14 of the 19 specimens being positive in dilutions of 1:8 or more. The infectious mononucleosis sera were less uniformly distributed; the possible significance of this is discussed below. Table II compares the distribution of the titers observed in 19 hepatitis patients with the distribution which might have been predicted from the frequencies found in the control group.

Discussion. These observations indicate that one of the results of viral hepatitis is an increase in the ability of the patient's serum to agglutinate the red cells of the rhesus monkey. We are not able to say whether this effect is due to the presence of a true antibody, to the presence of the virus itself, or whether the reaction is based upon the appearance of an abnormal serum constituent resulting from physiological disturbances related to the disease. A test such as we have described, if proven to be reliable, should have two important functions. First, it would be a valuable aid in the differential diagnosis of the jaundiced patient. In our limited series the individuals whose jaundice was related to hepatic disease other than viral hepatitis fell into the normal category insofar as the rhesus erythrocyte agglutina-

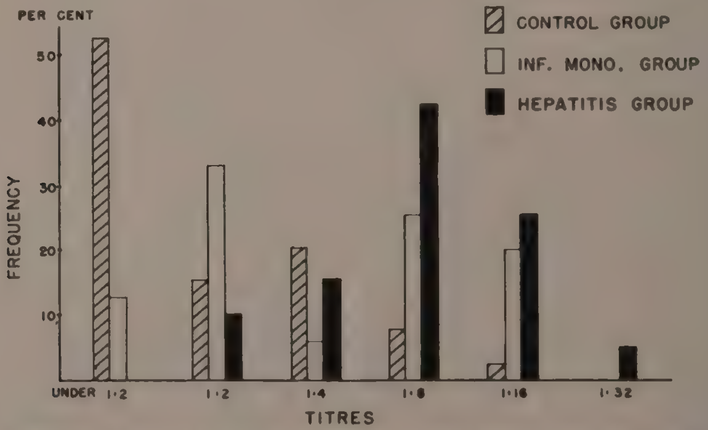

FIG. 1. Comparison of titres observed in control, infectious mononucleosis and viral hepatitis groups. The values represent the percentages of each group having the indicated titres.

TABLE II. Comparison of Expected and Observed Distribution of Titers in the Hepatitis Group.

	Titers					
	Under 1:2	1:2	1:4	1:8	1:16	1:32
Expected frequency based on control group	10	3.1	3.9	1.6	0.4	0
Observed frequency in hepatitis group	0	2	3	8	5	1

tion is concerned. Of the 19 cases of viral hepatitis, 14 showed elevated agglutinations titers. Thus, an agglutination titer of 1:8 or greater would support the diagnosis of viral hepatitis, while a titer less than 1:8 would argue against such a possibility. Since the test is positive (a titer of 1:8 or more) in almost one half of our cases of infectious mononucleosis. it cannot be said that it is a specific test for viral hepatitis. However, the liver is sometimes involved in infectious mononucleosis, so the positive tests obtained here may be related to hepatic disturbance. Furthermore, since infectious mononucleosis is ordinarily recognized by hematological and serological tests, the value of the rhesus erythrocyte agglutination reaction is not decreased by this finding.

A second possible field for use lies in the screening of donors to be used in the collection of whole blood or blood plasma. If the test were applied to the donors reported here, 15 of the 125 examined would have been rejected as possible asymptomatic carriers of viral hepatitis. Of course the evidence is purely presumptive, and further studies may show that such individuals may in fact not be carriers. However, since a substantial percentage of infected individuals in the viral hepatitis group (Gr. IV.) is detected by the test, its use as a screening device should be investigated. The fact that some infectious mononucleosis sera give positive reactions does not detract from its possible value as a screening procedure since it may be equally desirable to eliminate these individuals from the donor groups.

Summary. A hemagglutination test is described, wherein the sera of individuals diagnosed as having contracted viral hepatitis agglutinate the red blood cells of the rhesus monkey in dilutions of 1:8 or greater, as contrasted with the sera of a majority of apparently healthy blood donors and of jaundiced patients in whom a diagnosis of viral hepatitis was excluded. The observation, although made empirically and requiring confirmation, appears to offer promise and is being investigated at greater lengths.

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1. Neefe, J. R., Norris, R. F., Reinhold, J. G., Mitchell, C. B., Howell, D. S., and Murray, R., Diefenback, W. C. L., Ratner, F., Leone, N. C., and Oliphant, J. W., *J.A.M.A.*, 1954, v154, 1066.

2. Fitch, D. R., Watanabe, R. K., Kassouny, D., Neefe, J. R., Reinhold, J. G., and Norris, R., *Am. J. Clin. Path.*, 1955, v25, 158.

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Hypotensive Activity of Natural and Synthetic Estrogens in Metacorticoid Hypertensive Rats. (22815)

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Estrogens were reported some years ago to have a hypertensive action in rats(1,2); however, subsequent workers were unable to confirm such effects on the blood pressure of normal or renal hypertensive animals(3-7). On the contrary, several reports suggested an antihypertensive action of estrogens(8-10). The present paper confirms the latter in the case of the metacorticoid hypertensive rat treated with 3 natural and 2 synthetic estrogens.

Methods. The experimental animals consisted of 80 male, Sprague-Dawley rats that had received subcutaneously a 40 mg wax pellet containing 20 mg desoxycorticosterone acetate (DCA) at least 3 months previously and that had been maintained on 0.86% NaCl solution and Rockland Rat Diet *ad libitum*. Saline and food were not removed during the course of the tests. The systolic blood pressures of treated rats were estimated without anesthesia or heating using a photoelectric tensometer(11) before and 2, 4, and 6 hours after injection, and were compared to simultaneous readings from solvent-injected controls. Pressure readings on individual rats were made in ignorance of the injection the rat had received and of the previous pressure readings. Each test usually consisted of 4 treated and 4 control rats run simultaneously by one person on the same tensometer in a darkened room. The 5 estrogens listed in Table I were injected subcutaneously in a total of 40 rats as 1% solutions in corn oil at a dosage of 20 mg/kg. Injected material was always deposited at least 60 mm from the site of puncture in order to prevent leakage. The significance of *changes* in blood pressure, as compared to those in the controls, was estimated by the rank-sum method(12).

Results. The mean group blood pressures are listed in Table I. The 3 natural estrogens had a mild anti-hypertensive effect, while the 2 synthetic compounds were more potent. The fall in blood pressure after treatment with the epiestriol derivative was highly significant and reduced blood pressure to the normotensive range. From the subsequent condition of the animals and also from our experience with many other steroids, there was no reason to ascribe the hypotensive responses to a general toxic action.

The 3 natural estrogens were considerably more potent than the 2 synthetic compounds with regard to their metrotrophic, osteotrophic, and lipemic actions, as measured by uterine growth in immature mice(13), bone density in adult mice, and blood lipids in cholesterol-fed chicks, respectively.[*] This is in contrast to the greater hypotensive activity of the synthetic estrogens (Table I).

Summary. Three natural and 2 synthetic estrogens were compared for hypotensive activity in metacorticoid hypertensive rats. The most active compound was 16-epiestriol 3-methyl ether.

TABLE I. Effects of Estrogens in Metacorticoid Rats.

Compound	No. rats	Mean group blood pressure, mm Hg Before inj.	2 hr	4 hr	6 hr
Estrone	4	188	178	176	170*
Estradiol	8	185	172	173	164*
Estriol	8	171	174	163†	160*
16-epiestriol 3-methyl ether	8	186	143‡	139‡	130‡
16-oxoestradiol 3-methyl ether	12	180	165	162*	154†
Pooled controls	40	187	183	188	188

* P <.05. † P <.01. ‡ P <.001.

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[*] To be published by R. A. Edgren and D. L. Cook.

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1. Grollman, A., Harrison, T., and Williams, J., *J. Pharm. Exp. Ther.*, 1940, v69, 149.

2. Leathem, J., and Drill, V., *Am. J. Physiol.*, 1943, v139, 17.

3. Schroeder, H., *J. Exp. Med.*, 1942, v75, 513.

4. Matthews, D., Emery, F., and Weygandt, P., *Endocrinology*, 1943, v33, 177.

5. Hill, H., PROC. SOC. EXP. BIOL. AND MED., 1946, v63, 458.

6. Page, E., and Ogden, E., *Am. J. Ob. Gyn.*, 1947, v53, 150.

7. Wakerlin, G., and Gaines, W., *Am. J. Physiol.*, 1940, v130, 568.

8. Selye, H., *Rev. Canad. Biol.*, 1951, v9, 474.

9. Braun-Menendez, E., *Rev. Soc. Argent. Biol.*, 1952, v28, 23.

10. Stamler, J., *Circulation*, 1954, v10, 896 (and refs.).

11. Kersten, H., Brosene, W., Ablondi, F., and Subbarow, Y., *J. Lab. Clin. Med.*, 1947, v32, 1090.

12. Wilcoxon, F., *Biom. Bull.*, 1945, v1, 80.

13. Edgren, R. A., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 569.

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Some Aspects of Relationship between Antigens of *Pasteurella pestis* and *Pasteurella pseudotuberculosis*. (22816)

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Schütze(1) established that *Pasteurella pseudotuberculosis* and *P. pestis* shared a common somatic antigen. Bhagavan *et al.* (2) have recently confirmed Schütze's finding and have presented data indicating that 5 of 7 *P. pestis* antigens are shared with *P. pseudotuberculosis*. Fraction I of *P. pestis*, according to the authors, is not shared with *P. pseudotuberculosis*. Since the toxin of the plague bacillus is considered an endotoxin, as originally stated by Rowland(3), it can be postulated that as an antigen it would be located in the somatic complex. The study of plague toxin has, in fact, usually been preceded by some operation designed to rupture the cells, such as prolonged growth followed by treatment with toluene(4), alternate freezing and thawing(5,6), and neutral salt extraction of acetone-killed bacilli(7). Somatic antigens of *P. pestis* which are shared with *P. pseudotuberculosis* do not include the plague toxin (2,8,9). For this reason, the toxin of *P. pestis* may be defined as a somatic antigen which is not shared with *P. pseudotuberculosis*.

The present communication describes attempts to establish these criteria using a previously described gel-precipitin technic(10, 11) by comparing lysed and unlysed suspensions of *P. pestis* and lysed suspensions of *P. pestis* and *P. pseudotuberculosis*.

Methods and materials. (a) *Preparation of cell suspensions*: Organisms were grown on heart infusion agar (Difco) at 37°C and harvested in phosphate buffered saline at pH 7.0. Organisms used were *P. pestis* strain A1122 and *P. pseudotuberculosis* strain 1, received from Dr. S. F. Quan, Communicable Diseases Laboratory, USPHS, San Francisco, Calif. The final suspensions contained 1.97-2.00 mg total nitrogen/ml. (b) *Rupture of cells*: Suspensions described above were placed in the glass receiver tubes of the Mickle disintegrator(12). Approximately 5 g of "ballottini"(12) were added to 20-30 ml of organism suspension. The instrument was allowed to vibrate at its maximum amplitude for 15 minutes (time determined by preliminary trials to be described in the next section. (c) *Determination of patterns in 2-channel comparator cells*: The technic used was similar to that described previously(10,11), using 1:50 anti-plague serum globulin in 1% clarified agar as internal reactant(13). *Experi-*

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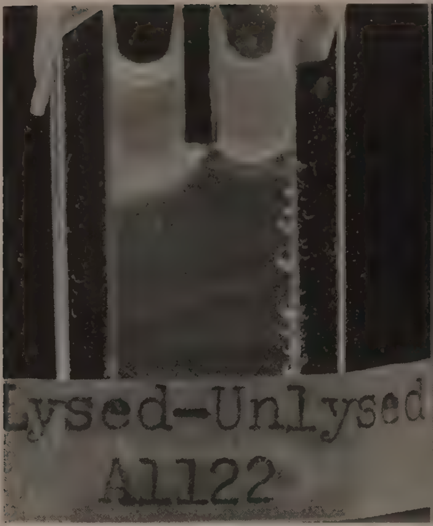

Fig. 1

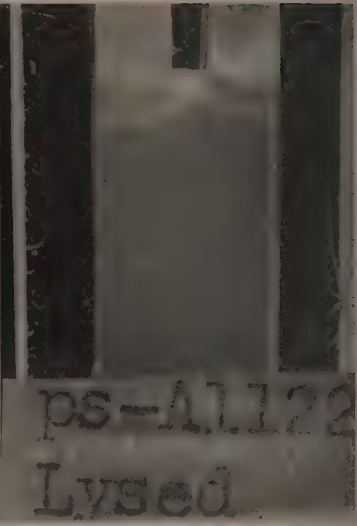

Fig. 2

FIG. 1. Antigen comparator cell showing match between a lysed suspension of *Pasteurella pestis* strain A1122 (on the left) and an unlysed suspension.

FIG. 2. Antigen comparator cell showing a match between lysed suspensions of *P. pseudotuberculosis* (left) and *P. pestis* strain A1122 (right). Approximately 2× enlargement.

mental observation. Three types of experiments were carried out and will be reported in this paper: (1) Lysis of suspensions of plague bacilli; (2) Comparison of lysed and unlysed suspensions with respect to antigenic components; (3) Comparison of lysed suspensions of *P. pestis* with those of *P. pseudotuberculosis*.

(1) *Lysis of suspensions of plague bacilli*: As mentioned above, suspensions of *P. pestis* strain A1122 were subjected to high speed vibration in the Mickle disintegrator in the presence of very small glass beads (ballottini). At 10-minute intervals specimens were withdrawn, the turbidity noted, and added to an aliquot of formalinized saline. Direct smears and cell counts using the Petroff-Hausser counting chamber, were made on the formalinized suspensions. The smears were Gram stained, density of cells and morphological characteristics were evaluated qualitatively. In general, high speed vibration produced with time a diminution in cell count accompanied by a decrease in turbidity of the cell suspensions. Approximately 90% of the reduction in cell count occurred during the first 15-20 minutes of shaking; subsequently, little change took place. The data indicated that this was due to an inherent inefficiency of the process with respect to small coccoid forms. Cultures of plague bacilli are usually mixtures of these and of larger bacillary forms. The disappearance of the latter was readily followed on the Gram stained smears. The action of high frequency vibration was to destroy selectively the larger forms.

(2) *Antigenic comparison of lysed and unlysed suspensions*: The mixture obtained by lysis in the Mickle disintegrator for 15 minutes was matched in 2-channel comparator cells against a suspension of identical composition except that it was not lysed. The test was set up in triplicate. The results of all 3 tests were essentially as shown in Fig. 1. Three rapidly moving components which were common to both preparations produced precipitin zones at considerable distance from points of origin. The fastest and the slowest of these components were present in similar concentration in both lysed and unlysed suspensions, as judged by the distance of their

precipitin zones from the meniscus. Since the distance of diffusion was not increased by lysis, these zones are marked as being of extracellular origin. The third component in this group was of intermediate mobility with respect to the other two, and its distance from the origin was increased on the side of the lysed suspension. The result, as shown in Fig. 1, was a zone of sharp equivalence point characteristics which curved upward from left to right of the comparator cell. The next two zones were common to both suspensions, but significantly increased in concentration in the lysed suspension, as shown by the greater distance of movement on the left side of the cell in Fig. 1. This condition may be explained by postulating 2 endo-antigens which are present in the intercellular fluid due to leakage or autolysis of some cells, but are markedly increased in concentration when all cells are ruptured. Finally, a broad 6th zone proximating the meniscus is present only on the side delivering material from the lysed suspension. This component must be an endo-antigen present in significant concentration only when most of the cells are lysed.

(3) *Antigenic comparison of lysed suspensions of P. pestis and P. pseudotuberculosis*: Lysed suspensions of *P. pestis* strain A1122 and *P. pseudotuberculosis* strain 1 were matched in 2-channel comparator cells as before. After 10 days, the appearance was that shown in Fig. 2. Visual and photographic observations confirmed the fact that the zones produced by the lysed *P. pestis* suspensions were identical to those produced in the preceding test, except for the presence of 3 instead of 2 zones in the intracellular antigen complex. (a) Two extracellular antigens appeared to be shared by both bacterial populations. These are the most rapidly moving zones which were present in lysed and unlysed *P. pestis* suspensions in like concentration. The third extracellular antigen of *P. pestis* was not shared with *P. pseudotuberculosis*, as indicated by the semi-radial pattern arising from the right (*P. pestis*) side of the cell. This precipitation zone had the same characteristics with respect to density as the antigen whose mobility was increased by lysis in the preceding test. (b) The next two intracellular antigens of *P. pestis* were not shared with *P. pseudotuberculosis*. (c) Finally, there was striking evidence that the slowly diffusing meniscus antigen of *P. pestis* is present in greater concentration in *P. pseudotuberculosis*.

Discussion. The finding of 7 antigens of *Pasteurella pestis* in the avirulent strain A1122 confirms the findings of Bhagavan *et al.* (2). However, it must be emphasized that this pattern does not represent a maximum or fixed number of possible components. Many of the variables involved have been mentioned by Oudin(14), Munoz and Becker (16), Jennings(15), and Ransom *et al.*(11).

The following description appears from this work to represent essentially the antigenic structure of *P. pestis* and *P. pseudotuberculosis*. There are at least 2 antigens common to both organisms which diffuse readily from the surface of the cell, which probably correspond to the "envelope" antigens of Schütze (loc. cit.). A third *P. pestis* antigen which diffuses quite readily from suspensions but is increased in concentration when the cells are disintegrated is not shared with *P. pseudotuberculosis*.

The finding of 2 shared "envelope" antigens between these two organisms confirms the observation of Larson *et al.*(17) that cross reacting antigens are present in ether-treated water-soluble preparations of the two organisms. Since Fraction I has been shown to be absent in *P. pseudotuberculosis*, this antigen is probably represented by the third zone of intermediate mobility referred to above.

There are 2 or 3 antigens which are so intimately associated with the soma that their appearance in the extracellular fluid is limited to relatively small quantities unless the cell is broken up. These *P. pestis* antigens are not shared with *P. pseudotuberculosis*.

There is at least one intracellular antigen which is so firmly bound to the soma that it does not appear in these tests at all unless the cell walls are very largely destroyed. This antigen is possessed also by *P. pseudotuberculosis*, and is perhaps identical with the so-

TABLE I. Distribution of Antigens in *Pasteurella pestis* and *P. pseudotuberculosis* as Revealed by the 2-Channel Comparator Cell.

Probable cellular location	Shared	*P. pestis* only	*P. pseudotuberculosis* only
Envelope	2	1*	0
Somatic	0	3†	0
Intracellular	1	0	0

* Fraction I? † Fraction II?

matic antigen of Schütze.

Since toxin is possessed by *P. pestis*, including strain A1122, but not by *P. pseudotuberculosis*, one of the unshared antigens most probably represents the toxin. Plague toxin is known to be firmly bound to the cell. The only likely candidates then, assuming that plague toxin is represented in these tests by one or more precipitation zones, are the two or three somatic antigens which *P. pestis* does not share with *P. pseudotuberculosis*. The hypothetical distribution of antigens between *P. pestis* and *P. pseudotuberculosis* is shown in Table I.

Summary. It has been shown that high speed vibration of cells of *P. pestis* in the presence of "ballottini" is accompanied by disintegration of the bacillary forms with concurrent release of antigenic material. At least one antigen was present in the lysed material which could not be demonstrated when cells were not lysed. 2. *P. pestis* and *P. pseudotuberculosis* appear to share 2 free "envelope" antigens as well as a single somatic antigen. In addition, it has been shown that *P. pestis* possesses a minimum of 1 "envelope" and 3 somatic antigens which are not shared with *P. pseudotuberculosis*. No antigens which could be considered specific for *P. pseudotuberculosis* were revealed.

1. Schütze, H., *Brit. J. Exp. Path.*, 1932, v13, 284.
2. Bhagavan, N. V., Chen, T. H., and Meyer, K. F., Proc. Soc. Exp. Biol. and Med., 1956, v91, 353.
3. Rowland, S., *J. Hyg.*, (*Camb.*), 1910, v10, 536.
4. Petrie, G. F., In: Gt. Britain, Med. Res. Council. *A system of bacteriology in relation to medicine*, 1929, v3, 137.
5. Girard, G., *Ann. Med.*, 1937, v42, 478.
6. ———, *Ann. Inst. Past.*, 1941, v67, 365.
7. Baker, E. E., Sommer, H., Foster, L. E., Meyer, E., and Meyer, K. F., *J. Immunol.*, 1952, v68, 131.
8. Schar, M., and Thal, E., Proc. Soc. Exp. Biol. and Med., 1955, v88, 39.
9. Chen, T. H., and Meyer, K. F., *J. Immunol.*, 1955, v74, 501.
10. Ransom, J. P., Quan, S. F., Hoggan, M. D., and Omi, G., Proc. Soc. Exp. Biol. and Med., 1955, v88, 173.
11. Ransom, J. P., Quan, S. F., Omi, G., and Hoggan, M. D., *J. Immunol.*, 1955, v75, 265.
12. Mickle, J., *Roy. Microscop. Soc.*, 1948, v68, 10.
13. Lamanna, C., and Mallette, M. F., *J. Bact.*, 1954, v67, 503.
14. Oudin, J., In: *Methods in Medical Research*, 1952, v5, 335.
15. Jennings, R. K., *J. Bact.*, 1954, v67, 565.
16. Munoz, J., and Becker, E., *J. Immunol.*, 1950, v65, 47.
17. Larson, C. L., Philip, C. B., Wicht, W. C., and Hughes, L. E., *ibid.*, 1951, v67, 289.

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Comparison of Noludar (Methylprylon) and Pentobarbital on Respiratory Minute Volume in Rabbits.[*][†] (22817)

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Noludar (3,3-diethyl-2, 4-dioxo-5-methyl piperidine),[‡] has been demonstrated in both animal and clinical trials to be a mild hypnotic and sedative with very few side effects (1,2). It has been suggested clinically that Noludar, when given with morphine, does not add to respiratory depression produced by the opiate and hence would be a desirable agent to be used when combination therapy is necessary.[§] The purpose of this study was to compare respiratory depressant effects of Noludar and pentobarbital alone and in combination with morphine on rabbit respiratory minute volume.

Methods. Rabbits, ranging in weight from 1 to 2 kg, were used. Respiratory minute volumes were determined in 2 L. spirometer which collected expired air by means of a Lucite mask fitted with one-way flutter valves. In each experiment, at least 3 control values were determined before injection of the drug. Following control determinations, the drugs, dissolved in normal saline, were injected into marginal ear vein. When morphine was used it was always injected before the hypnotic drug. Minute volume measurements were then taken every 15 minutes for a 2-hour period after injection. Respiratory minute volume depression was expressed as a percentage of control and these percentages summed for the 2-hour period to yield 2-hour total area values(3). All comparisons were made from these values.

Results. Fig. 1 represents the calculated regression plots for morphine (M), Noludar (N), pentobarbital (P) and combinations of

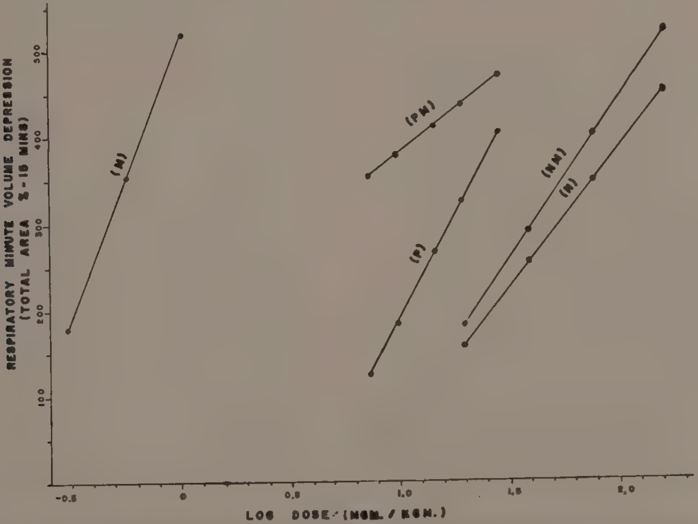

FIG. 1. Calculated regression plots for M (morphine), P (pentobarbital), N (Noludar), NM (Noludar-morphine) and PM (pentobarbital-morphine).

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[‡] We wish to thank Dr. M. J. Schiffrin for the Noludar.

[§] Unpublished communication.

TABLE I. Regression Statistics for Regression Lines Fig. 1.

Drugs	No. animals/regression line	a*	b	$\bar{x}$†	Linearity of regression F	Significance P
Morphine (M)	18	360.56	566.12	.199	54.66	.001
Pentobarbital (P)	25	271.16	471.35	1.19	27.82	"
Noludar (N)	26	269.62	318.77	1.66	55.53	"
Morphine-pentobarbital (MP)	25	411.64	191.75	1.18	6.66	.05–.01
Morphine-Noludar (MN)	22	336.37	364.91	1.73	35.83	.001

* % total area (15 min. for 2 hr). † Log dose.

Regression equation, $Y = a + b\,(x - \bar{x})$.

pentobarbital (PM) and Noludar (NM) with 0.608 mg/kg of morphine. The data in each case were tested for linearity of regression and in all cases were found to be significantly linear. These regression statistics (4) are collected in Table I.

Comparisons of slopes of regression lines were conducted by means of t test of regression coefficients. Morphine had a significantly greater slope than Noludar ($t_{(42)} = 0.01 > p > 0.001$), than pentobarbital-morphine combination ($t_{(39)} = 3.50 p < 0.001$) and the Noludar-morphine combination ($t_{(36)} = 2.00\ 0.05 > p > 0.02$). The slope of pentobarbital alone was greater than that of pentobarbital-morphine combination ($t_{(46)} = 2.41\ 0.02 > p > 0.01$). Morphine when compared to pentobarbital and Noludar when compared to pentobarbital or Noludar-morphine were not found to be significantly different. The slope of Noludar-morphine, although somewhat greater than that of pentobarbital-morphine, was not significantly greater ($t_{(43)} = 1.80\ 0.10 > p > 0.05$).

The differences in slope among the various drugs and combinations do not allow direct comparisons of potency. We have therefore compared the differences between low and high doses of pentobarbital alone and with morphine and similarly low and high doses of Noludar alone and with morphine. These comparisons are detailed in Table II. Noludar-morphine combinations do not differ significantly from Noludar alone at either high or low doses. Pentobarbital-morphine combinations are, however, significantly more depressant than pentobarbital alone at low dose but not at high doses.

TABLE II. Comparison of Low (N–NM), High (N–NM), Low (P–PM) and High (P–PM).

Log dose (x)	Comparison	$t_{(df)}$	Significance P
.933	P_1–PM_1	$6.01_{(46)}$	.001
1.569	P_2–PM_2	$.74_{(46)}$	.50–.40
1.285	N_1–NM_1	$.58_{(44)}$	.60–.50
2.226	N_2–NM_2	$1.42_{(44)}$	.20–.10

TABLE III. Effect of Intermittent Injections of Pentobarbital and Noludar in Normal and Morphine-Treated Rabbits.

No. of doses of depressant	Morphine pretreatment, mg/kg	Noludar* treatment† effect
6	0	Survived
8	0	"
7	.608	Died 60 min. post-inj.
8	.608	Survived
4	1.220	"
7	1.220	"
8	1.220	Died 60 min. post-inj.

* All pentobarbital animals died at end of last dose.

† Each pentobarbital dose contained 10 mg/kg and each Noludar dose 35.5 mg/kg as calculated from regression lines as producing equivalent respiratory minute volume depression. Inj. rate was 1 dose/min.

In view of the apparent lack of difference between pentobarbital and Noludar at high doses, an attempt was made to compare pentobarbital and Noludar as to toxicity (*i.e.* cessation of respiration). Simultaneous intermittent injections of equidepressant doses of Noludar and pentobarbital were given to rabbits with and without morphine pretreatment. The results are summarized in Table III.

Discussion. There is some indication from the toxicity data that Noludar is less toxic than pentobarbital at equally depressant doses of the hypnotics alone and in combination with morphine. However, in evaluating these data, it must be considered that Noludar is a much longer acting hypnotic than pentobarbital and hence discrepancies in the time necessary to produce respiratory arrest might be expected. Nevertheless, 4 out of 7 animals survived for 4 hours or longer following Noludar while all 7 animals receiving equi-depressant doses of pentobarbital expired.

Summary. Noludar and pentobarbital alone and in combination with morphine were compared as to their respiratory minute volume depressant effects in rabbits. No statistically significant difference could be found between Noludar alone and in combination with 0.603 mg/kg of morphine. Pentobarbital, on the other hand, was significantly more depressant in combination with morphine at low pentobarbital doses but not at anesthetic levels.

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1. Pellmont, V. B., Studer, A., and Jurgens, R., *Schweiz. Med. Wochensch.* 1955, v85, 35.

2. Cass, L. J., Frederick, W. S., and Andosca, J. B., *New England J. M.*, 1955, v253, 586.

3. Yim, G. K. W., Keasling, H. H., Gross, E. G., and Mitchell, C. W., *J. Pharm. and Exp. Therap.*, 1955, v115, 96.

4. Mather, K., *Statistical Analysis in Biology*, Interscience Pub., New York, 1947.

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Carbohydrate Metabolism and Phosphate Turnover Rate in Scorbutic Guinea Pigs. (22818)

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Metabolism of carbohydrate is altered in ascorbic acid deficiency. Banerjee *et al.*(1-4) and Bacchus and Heiffer(5) observed decreased glucose tolerance in scorbutic guinea pigs. Murray *et al.*(6) and Stewart *et al.*(7) reported diminished glucose tolerance in scorbutic monkeys. Liver glycogen content was lower in such animals than in paired fed normal controls. Banerjee *et al.*(3,8,9) ascribed the decreased carbohydrate metabolism to diminished insulin content of the pancreas in scurvy. Recently Banerjee and Ghosh(10) reported diminished hepatic and muscle hexokinase activity in scorbutic guinea pigs possibly due to decreased insulin synthesis and supply in such animals. Insulin was shown to stimulate hexokinase(11) and reduce liver glucose-6-phosphatase activities(12). Considering the possible role of ascorbic acid in the biosynthesis of insulin and the functions of insulin at these levels of glucose metabolism, the glucose-6-phosphatase activity was determined in tissues of scorbutic and paired fed normal guinea pigs. Decrease in hexokinase activity and increase in glucose-6-phosphatase activity might consequently lead to diminished phosphorylated intermediates of the glycolytic system. The changes in phosphorylation and dephosphorylation processes might eventually alter inorganic phosphorus content of tissues in scurvy. Studies were, therefore, carried out to elucidate the pattern of metabolic changes in relation with carbohydrate metabolism in scorbutic guinea pigs. The hexokinases are known to be sulfhydryl enzymes, and it is now well accepted that ascorbic acid protects and maintains the sulfhydryl groups of the enzymes(13). As an introduction to further study on the role of ascorbic acid in carbohydrate metabolism, fructokinase activity of the brain tissue of scorbutic and paired fed normal guinea pigs was determined.

Methods. Female well-growing guinea pigs of weights varying between 230 and 260 g were distributed into 16 pairs. Feeding a scorbutic diet, with or without supplement of ascorbic acid, by paired feeding technic and care of animals were the same as described previously(14). Pairs of animals were sac-

TABLE I. Effect of Scurvy on Inorganic Phosphorus Values of Blood and Liver of 10 Guinea Pigs in Each Series.

	mg P/100 ml blood	mg P/100 g liver
Normal	4.7 ± .25*	34.1 ± 2.1
Scorbutic	6.2 ± .50	46.1 ± 1.2
t	2.5	4.8

* Stand. error.

rificed after an over-night fast on 25th day of experiment, and inorganic phosphorus contents of blood and liver, glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate contents of liver, glucose-6-phosphatase activity of liver and kidney and fructokinase activity of brain were determined. Total weights of livers of some pairs of guinea pigs were recorded and the values calculated per 100 g body weight. For estimation of *inorganic phosphorus,* blood was collected in an oxalated tube and 1 ml aliquot was analysed by the method of Fiske and SubbaRow (15). For liver inorganic phosphorus and phosphorylated intermediates, the tissue was immediately frozen after killing the animal and was extracted with ice cold 10% trichloroacetic acid. A portion of the extract was neutralized, in cold, to phenolphthalein and inorganic phosphate was precipitated with magnesia mixture over night and then estimated. Another aliquot was analysed for glucose-6-phosphate, fructose-6-phosphate and fructose-1, 6-diphosphate according to the methods described by LePage and Umbreit(16). Results are given in Tables I and II. For estimation of *glucose-6-phosphatase* and *fructokinase* activity, tissues were removed from body immediately after killing the animal and cooled in cracked ice for 3-5 minutes. Glucose-6-phosphatase activity of liver and kidney was assayed by the method of Swanson(17). The tissues were homogenized with exactly 3 volumes of ice-cold 0.25 M sucrose in an ice-cold all glass homogeniser. 0.1 ml of this homogenate, 0.1 ml of 0.04 M glucose-6-phosphate (pH 6.5) and 0.1 ml of 0.1 M citrate buffer of pH 6.5 were mixed in 1 cm diameter duplicate tubes, incubated for 30 minutes at 37° and the reaction terminated by addition of 0.3 ml of 10% trichloracetic acid. In control tubes, water was added in place of the substrate. The samples were diluted to 3 ml, centrifuged and analysed for inorganic phosphorus (15). Dry weights of tissues were obtained from 1 ml homogenate, less the weight of sucrose present. Inorganic phosphorus liberated by the enzymatic hydrolysis in one hour per mg dry tissue is presented in Table III.

TABLE II. Effect of Scurvy on Phosphorylated Intermediate Contents of Liver of Guinea Pigs (mg/100 g Fresh Liver). 6 animals/series.

	Glucose-6-phosphate	Fructose-6-phosphate	Fructose-1,6-diphosphate
Normal	1166 ± 41	17.0 ± 1.1	20.2 ± .97
Scorbutic	995 ± 44	11.1 ± 1.5	15.7 ±1.0
t	2.7	3.2	3.4

TABLE III. Effect of Scurvy on Glucose-6-phosphatase Activity of Tissues of Guinea Pigs (γ P Liberated/mg Dry Tissue/ Hr). 8 animals/series.

	Liver	Kidney
Normal	27.4 ± 4.2	31.0 ± 5.5
Scorbutic	40.5 ± 4.5	36.8 ± 15.0
t	2.1	.7

For determination of *fructokinase activity,* brain tissue was homogenized in an ice-cold all glass homogeniser with 4 volumes of ice-cold 0.15 M potassium phosphate buffer of pH 7.5 containing 0.15 M potassium fluoride. Fructose phosphorylation studies were studied in an incubation medium proposed by Vestling *et al.*(18), using 1 ml portions. Incubation was carried out for 30 minutes at 37° and the reaction stopped by addition of 1 ml of 0.3 N barium hydroxide, followed by 1 ml of 5% zinc sulfate and water to 10 ml, mixed and filtered. Control experiments were conducted using complete medium with omission of adenosinetriphosphate. Fructose disappearance was measured by the method of Roe(19). 1 ml homogenate was dried at 105° to constant weight, and weight of salts deducted to get dry weight of tissue. Fructose disappeared/mg dry tissue/hour is presented in Table IV.

Results. A change in tissue phosphorus content in scurvy is apparent from Table I. Though there is a significant increase in inorganic phosphorus content of blood, only 5 pairs out of 10 studied show this change. A

TABLE IV. Effect of Scurvy on Fructokinase Activity of Brain of 10 Guinea Pigs per Series. (γ fructose disappeared/mg dry tissue/hr.)

Normal	Scorbutic	t
123.3 ± 12	100.5 ± 35	.6

significant increase in liver inorganic phosphorus content in scurvy was observed. This increased inorganic phosphorus level of blood and liver in scorbutic animals suggests that either the mechanism responsible for removal of free phosphate is inefficient, or that hydrolysing the organic phosphates, *i.e.*, phosphatase activity, is increased.

We reported that hepatic and muscle hexokinase activity is diminished in scorbutic guinea pigs(10). Table III shows that glucose-6-phosphatase activity of the hepatic tissue is increased in scurvy. These changes are those which would be expected if an insulin insufficiency occurred in such animals. As a consequence it is likely that inorganic phosphorus level of tissues will tend to increase and glucose-6-phosphate content of liver would diminish in scurvy. The data presented in Table II clearly indicate that this ester content of livers of scorbutic guinea pigs is significantly decreased. This table further shows that fructose-6-phosphate and fructose-1, 6-diphosphate levels of livers in scurvy are lowered.

Table III shows that the hepatic glucose-6-phosphatase activity is increased in scurvy, and remains unchanged in the kidney. Langdon and Weakley(12) reported that alloxan diabetes in rats is accompanied by a more than 2-fold increase in hepatic content of the enzyme. In scurvy, however, there is comparatively less change, which could be due to a smaller decrease in insulin synthesis in scurvy than in alloxan diabetes. These findings, therefore, suggest that the quantities of phosphorylated intermediates turned over in unit time are reduced below normal and further add to the evidence that the defect in carbohydrate metabolism in scurvy is quantitative rather than qualitative. It is, however, difficult to assess whether these quantitative changes in the phosphorylation cycle are of sufficient magnitude to account for the disturbed glucose tolerance in scorbutic animals.

No change in fructokinase activity in the brain of guinea pigs has been observed in scurvy. Table IV shows that, though there is a tendency to decrease, in some scorbutic animals, the individual variations are too great to make the change in average values significant. Fructose phosphorylation is also not hampered in insulin insufficiency due to alloxan diabetes(19-21). It has been observed that in scorbutic guinea pigs the weights of livers were considerably increased. The values calculated in g/100 g body weight of normal and scorbutic guinea pigs are 2.8 ± 0.2 and 4.8 ± 0.4 respectively. If the inorganic phosphorus and glucose-6-phosphatase activity of livers of scorbutic guinea pigs is expressed on the basis of total liver, these values will be further increased.

Summary. Inorganic phosphorus content of blood and liver and hepatic glucose-6-phosphatase activity are increased in scorbutic guinea pigs. Glucose-6-phosphate, fructose-6-phosphate and fructose-1,6-diphosphate contents of liver are decreased in scorbutic guinea pigs. Fructokinase activity of brain remains unchanged in scorbutic guinea pigs. Considerable increase in weight of livers of scorbutic guinea pigs has been observed.

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1. Banerjee, S., *Ann. Biochem. Exp. Med.*, 1943, v3, 157.

2. ———, *ibid.*, 1943, v3, 165.

3. Banerjee, S., and Ghosh, N. C., *J. Biol. Chem.*, 1946, v166, 25.

4. ———, *ibid.*, 1947, v168, 207.

5. Bacchus, H., and Heiffer, M. H., *Am. J. Physiol.*, 1954, v176, 262.

6. Murray, H. C., and Morgan, A. F., *J. Biol. Chem.*, 1946, v163, 40.

7. Stewart, C. T., Salmon, R. J., and May, C. D., *Am. J. Dis. Child.*, 1952, v84, 677.

8. Banerjee, S., *Nature*, 1943, v152, 329.

9. ———, *ibid.*, 1944, v153, 344.

10. Banerjee, S., and Ghosh, P. K., PROC. SOC. EXP. BIOL. AND MED., 1955, v88, 415.

11. Price, W. H., Cori, C. F., and Colowick, S. P., *J Biol. Chem.*, 1945, v160, 633.

12. Langdon, R. G., and Weakley, D. R., *ibid.*, 1955, v214, 167.

13. Mapson, L. W., *Vitamins and Hormones*, 1953, v11, 10.

14. Banerjee, S., and Deb, C., *J. Biol. Chem.*, 1951,

v190, 177.

15. Fiske, C. H., and SubbaRow, Y., *ibid.*, 1925, v66, 375.

16. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., *Manometric Techniques and Related Methods for Study of Tissue Metabolism,* Burgess Publishing Co., Minneapolis, 1945, p159.

17. Swanson, M. A., *J. Biol. Chem.*, 1950, v184, 647.

18. Vestling, C. S., MylRoie, A. K., Irish, U., and Grant, N. H., *ibid.*, 1950, v185, 789.

19. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1928, v76, 755.

20. Miller, M., Drucker, W. R., Owens, J. E., Craig, J. W., and Woodward, H., Jr., *J. Clin. Invest.*, 1952, v31, 115.

21. Chernick, S. S., and Chaikoff, I. L., *J. Biol. Chem.*, 1951, v188, 389.

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Enzymatic Estimation of Urinary Steroids.* (22819)

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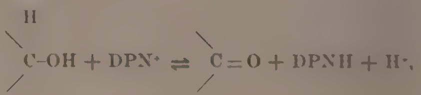
Existing procedures for the determination of urinary steroids depend upon either chemical reactions or bioassay. Despite great interest in this subject and its clinical importance, simple and specific micro-methods for the estimation of the amounts and types of urinary steroids have not been perfected(1). The novel procedure to be described here permits the simple microestimation of certain types of steroids in urine. This procedure depends upon selective enzymatic oxidations and reductions of steroids and the spectrophotometric measurement of the associated changes in the concentrations of diphosphopyridine nucleotide coenzymes. Enzymatic estimations are characterized by a high degree of specificity and sensitivity. It is thus possible to obtain quantitative information on the principal types of steroid ketones and alcohols present in a few milliliters of urine without resorting to tedious isolation and separation techniques. *Pseudomonas testosteroni* is a soil bacterium capable of growing on testosterone and related steroids as its only carbon source, and of oxidizing such steroids to carbon dioxide and water(2). The presence of steroids in the growth medium of this bacterium causes the induction of certain diphosphopyridine nucleotide-linked enzymes which reversibly interconvert particular hydroxy- and ketosteroids with a high degree of steric specificity(2-4). These enzymes have been named *hydroxysteroid dehydrogenases,* and their potential usefulness for the micro-estimation of steroids was pointed out in 1952. Subsequently, steroid dehydrogenases have been applied to the measurement and identification of single steroids(2-5). The purification and substrate specificities of 2 enzymes suitable for analytical purposes have been described(5-7). Briefly stated, these enzymes are: 1) a *3α-hydroxysteroid dehydrogenase* (designated α enzyme) which catalyzes the reversible oxidation of 3α-hydroxysteroids of the C-19 and C-21 groups; and 2) a *3β- and 17β-hydroxysteroid dehydrogenase* (designated β enzyme) which catalyzes the reversible oxidations of 3β- and 17β-hydroxysteroids to their respective ketones. Thus, the specificity of these enzymes fortunately coincides with the principal types of urinary steroids *i.e.* 17-ketosteroids as well as 3α-hydroxy- and 3β-hydroxysteroids of both the C-19 and C-21 series. The *analytical procedure* depends upon interconversions of steroid alcohols and ketones according to the general equation:



$$\overset{H}{>C{-}OH} + DPN^+ \rightleftharpoons {>C{=}O} + DPNH + H^+,$$

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when DPN^+ and DPNH refer to the oxidized and reduced forms of diphosphopyridine nucleotide respectively. DPNH has an absorption maximum ($\epsilon = 6{,}220$) at the wavelength of 340 mμ, where DPN^+ has negligible absorption. Thus, the increase in absorption at 340 mμ in the forward reaction is an accurate measure of the amount of DPNH produced and hence of the amount of steroid alcohol oxidized. Conversely, the decrease in absorption of DPNH in the *back* reaction is a measure of the amount of ketosteroid reduced. An accurate estimation of the total amounts of steroids present requires conditions under which the reaction equilibria are displaced either completely to the right (forward reaction) or to the left (back reaction), even in the presence of relatively large quantities of reaction products.

Quantitative conversions in the forward reaction were achieved by raising the pH to 9.5 and incorporating hydrazine as a ketone binding reagent into the reaction mixture. The back reaction is favored by lowering the pH, but only limited use can be made of this because of the instability of DPNH below pH 5.5. Complete reduction of ketosteroids to steroid alcohols was obtained if diphosphopyridine nucleotidase (DPN-ase) from zinc deficient *Neurospora*(9) was added to the reaction system. This enzyme destroys the DPN^+ formed during the back reaction by rupture of the nicotinamide-ribose bond of DPN^+, but does not attack DPNH.

Materials and methods. Extracts of human urine were prepared from 25 ml aliquots of 24 hour collections. Each aliquot was subjected to hydrolysis of steroid conjugates by incubation with beef liver β-glucuronidase (1000 units(10) per ml of urine) at pH 4.5 and 30° for 24 hours. Following extraction with three 15 ml portions of CH_2Cl_2, the aqueous residue was acidified to pH 1 with H_2SO_4 and further incubated at 30° for 24 hours. The residue was then reextracted with three 15 ml portions of CH_2Cl_2 and the combined solvent extracts evaporated to dryness. All evaporations were carried out in a rotating evaporator *in vacuo* below 45°. The residue was dissolved in methanol and passed over a 70 × 10 mm column of Amberlite MB 1, a strong base-strong acid, anion-cation mixed bed exchange resin obtainable from the Rohm and Haas Co., Philadelphia. The resin was well washed with water and methanol before use. The ion exchange resin quantitatively removes phenolic estrogens and eliminates other ionic constituents. The extract was evaporated, redissolved in 70% aqueous methanol and partitioned against *n*-hexane. The methanolic layer was evaporated to dryness and the residue dissolved in 0.50 ml methanol. Aliquots of this extract were then subjected to enzymatic reaction first with α enzyme and then with β enzyme in both forward and back reactions. The preparation of the enzymes has been described(7). All reactions were carried out in a Beckman DU spectrophotometer in cuvettes of 1 cm light path at approximately 25° and 340 mμ. The system for the *forward reaction* contained in a total volume of 3.0 ml: 100 μmoles of sodium pyrophosphate buffer pH 9.5, 0.5 μmole DPN^+, 1.0 millimole hydrazine sulfate (adjusted to approximately pH 9.5) and 0.025 to 0.075 ml of urine extracted in 0.1 ml CH_3OH. Initial optical density readings were taken against a control cuvette containing all ingredients except urine extract. 1000 units α enzyme in 0.01-0.02 ml were then added and readings continued until there was no further change in optical density for 15 minutes. 1000 units of β enzyme were then added and readings continued again to equilibrium. In the *back reaction*, the cuvettes contained: 100 μmoles orthophosphate buffer pH 5.5, 0.12 μmoles DPNH, 200 units DPN-ase and 0.025 to 0.050 ml of urine extract in 0.1 ml CH_3OH. Initial readings were taken at 340 mμ against a control cell containing no urine extract and no DPNH. An additional control containing only DPNH and enzymes was included, to compensate for small decreases in optical density of DPNH occurring during the course of the reaction. α and β enzymes (1000 units of each) were added successively to the reaction cuvettes and measurements made periodically until equilibrium was reached with each enzyme. The measurements were based

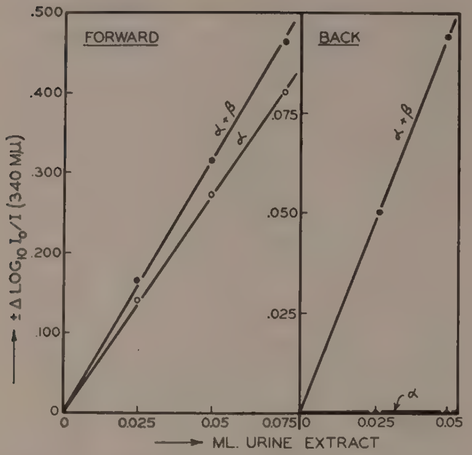

FIG. 1. Enzymatic assay of urinary steroid excretion in a 24-year-old healthy man whose output was 1350 ml in 24 hr. A 25 ml portion of urine was used to prepare 0.5 ml extract in CH_3OH as described in the text. Ordinate of the left graph shows the increases in optical density at equilibrium in the *forward reaction*, whereas that of the right graph shows the *back reaction*, in which decreases in optical density at equilibrium are plotted. Measurements were made with different amounts of urine extract and demonstrate linearity between the amount of pyridine nucleotide reduced or oxidized and vol of urine extract.

on the changes in optical density when each enzymatic reaction was complete.

Results. Typical measurements on an extract of male urine are shown in Fig. 1. Preliminary values for the steroid excretion of a small number of young subjects believed to be in good health are shown in Table I. The reliability of the assay procedure was established by several criteria: 1) A linear relation between the amount of extract and the change in optical density may be seen in Fig. 1, and has been repeatedly observed. 2) Various pure steroids added to a urine extract have been quantitatively estimated with a high degree of accuracy. The results of a recovery experiment of this type are shown in Table II. Suitable aliquots of a hydrolyzed urine extract and of solutions of purified androsterone and *epi*androsterone were each assayed successively with α and β enzymes in the *forward* reaction. The respective amounts of hydroxysteroids found with each enzyme are shown in experiments 1, 2 and 3 of Table II. A mixture of androsterone and *epi*androsterone was then assayed in the same manner and agreed within 1 percent or less with the individual assays obtained for each steroid separately (experiment 4). Similar aliquots of androsterone and *epi*androsterone were then measured in the presence of the same aliquot of urine extract and here also the recovery was better than 1% (experiment 5). Similar recovery experiments utilizing the *back* reaction have shown a recovery of *epi*androsterone measured as a 17-ketosteroid of within 1 to 2%. These experiments demonstrate the high accuracy of the method and excellent recoveries of physiological amounts of added steroids. 3) Some of the most abundant urinary steroids were added to an aliquot of urine which was then

TABLE I. Urinary Steroid Excretion in Micromoles per 24 Hours in Normal Young Adults.

Steroid groups measured	Men	Women	Examples of principal types of compounds measured
3α-Hydroxyl	54.6 (43.3 to 70.7)	34.8 (16.4 to 61.6)	Androsterone Etiocholan-3α-ol-17-one Pregnane-3α,17α,21-triol-11,20-dione Pregnane-3α,11β,17α,21-tetrol-20-one
3β-Hydroxyl + (17β-Hydroxyl)	8.86 (5.20 to 13.7)	5.98 (2.61 to 8.05)	*Epi*androsterone Dehydro*epi*androsterone
3-Ketone	<.5	<.5	Androstane-3,17-dione Etiocholane-3,17-dione
17-Ketone	21.4 (17.9 to 25.5)	9.71 (4.67 to 15.0)	Androsterone Etiocholan-3α-ol-17-one *Epi*androsterone Dehydro*epi*androsterone 4-Androstene-3,17-dione

Avg values are based on determinations in 6 men (age range 22 to 33 years) and 6 women (age range 23 to 37 years). Figures in parentheses refer to ranges of values in each group.

TABLE II. Estimation of Pure Steroids in the Presence of a Urine Extract.

Exp. No.	Material*		μmoles of hydroxysteroids		
		ml	α	β	$\alpha+\beta$
1	Urine extract	.05	.0580	.00725	.0653
2	Androsterone	.02	.0237	.000	.0237
3	*Epi*androsterone	.02	.000	.0435	.0435
4	Androsterone + *Epi*and'st'one	.02 .02	.0237	.0430	.0667
5	.05 ml urine extract + .02 ml androsterone + .02 ml *epi*androsterone		.0813	.0498	.131

* Urine extract was prepared from male urine as described in text (except that 1 ml extract was equivalent to 15 ml urine). Androsterone (m.p. 184.1-185.2° corr.) solution contained 3.5 mg in 10 ml methanol. *Epi*androsterone solution (m.p. 173-175° corr.) contained 6.5 mg in 10 ml methanol. Steroids were weighed to ± 0.1 mg. Values in table indicate that steroids were 94 to 96% pure by enzymatic assay.

carried through the entire procedure of hydrolysis and preparation of an extract as described. By enzymatic assay the recovery of androsterone was 94.5%, and that of pregnane-3α,17α,21-triol-11, 20-dione was 93.5%. 4) Duplicate assays on different aliquots of the same urine agreed within 5% or better.

Discussion. Several advantages characterize these enzymatic methods as compared to chemical and bioassay procedures. Sensitivity and specificity are inherent in enzymatic assays. The measurements depend upon specific changes in light absorption caused by DPNH, a compound of known structure, spectral characteristics and extinction, and may therefore be related directly to micromolar concentrations. These changes are produced by addition of a few micrograms of enzyme protein, which have negligible absorption at 340 mμ, and which produce no unspecific absorption changes or "background colors" at this or other wavelengths. Furthermore, unlike many chemical methods such as the Zimmerman reaction, equal amounts of different steroids give equivalent extinctions when measured by these enzymatic methods.

The sensitivity of the assay system depends upon the size of the reaction volume and the sensitivity of the spectrophotometer. Using microcells and a Beckman DU Spectrophotometer, 0.2 to 0.5 μg of steroid may be assayed with accuracy of a few per cent in a reaction volume of 0.2 ml. This sensitivity approaches that required for the measurement of the small amounts of steroids present in other body fluids such as the blood.

It is difficult to make comparisons between the magnitudes of the values shown in Table I and those available from other methods, because comparable measurements are available only for 17-ketosteroids. The values for 17-ketosteroids in normal young subjects by the enzymatic method are about 6 mg per day for men and 3 mg per day for women (assuming an average molecular weight of about 290). The respective ranges reported in the literature are 3 to 22 mg and 6 to 25 mg respectively(11). The enzymatic values are therefore consistent with the lower values obtained by the Zimmerman reaction, which are believed by some authors to be the most reliable(1,11).

Summary. A novel method for the sensitive and specific microestimation of steroids has been described. This method depends upon the selective oxidation or reduction of respectively hydroxy- and ketosteroids by highly purified hydroxysteroid dehydrogenases of bacterial origin. The method permits the estimation of 3α-hydroxysteroids, 3β-(and 17β-) hydroxysteroids, 3-ketosteroids and 17-ketosteroids.

The authors are indebted to Warner-Chilcott Laboratories, New York, for supplying "Ketodase" (β-glucuronidase) and to Syntex S. A. Mexico, for gifts of various steroids.

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1. For review see Conference on "Methods of Steroid Determination in Blood and Urine", *Recent Progress in Hormone Research, IX,* 1954; and critical appraisal by Marrian, G. F., *Proc. of the Third Intern. Congress Biochem.,* Brussels, 1955, p511.

2. Talalay, P., Dobson, M. M., and Tapley, D. F., *Nature,* 1952, v170, 620.

3. Talalay, P., and Dobson, M. M., *J. Biol Chem*, 1953, v205, 823.

4. Talalay, P., and Marcus, P. I., *Nature,* 1954, v173, 1189.

5. ———, *J. Biol. Chem.,* 1956, v218, 675.

6. Marcus, P. I., and Talalay, P., *Proc. Roy. Soc.*

London B, 1955, v144, 116.
7. ———, *J. Biol. Chem.*, 1956, v218, 661.
8. Barton, D. H. R., *J. Chem. Soc.*, 1953, 1027.
9. Kaplan, N. O., in Colowick, S. P. and Kaplan, N. O., eds., *Methods in Enzymology*, 1955, vII, 664, Academic Press, New York.
10. Talalay, P., Fishman, W. H., and Huggins, C., *J Biol. Chem.*, 1946, v166, 757.
11. Mason, H. L., and Engstrom, W. W., *Physiol. Revs.*, 1950, v30, 321.

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Trace Metal Requirements of Azotobacter.* (22820)

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Although metallic ion requirements for fixation of atmospheric nitrogen by the azotobacter have been studied for a number of years(1,2,3) an extension and reevaluation of the earlier work was thought worthwhile in light of recent advances in methodology of trace metal studies. These earlier studies established marked differences in response of both strains and species of the organism. Therefore, an added reason was to determine trace metal requirements of our strain *Azotobacter vinelandii* O which is used in many biochemical and bacteriological laboratories.

Materials and methods. Azotobacter vinelandii O was maintained and studied in Burk's sucrose mineral salts medium(4) in 500 ml Erlenmeyer shake flasks. The cultures were incubated at 30°C in Brunswick rotary shaker at 400 rpm. Flasks were covered with 50 ml pyrex beakers instead of cotton plugs. All glassware was cleaned by washing with detergent followed by 4 rinses in distilled water. It was then filled with 0.5% solution of tetra sodium salt of ethylene diamine tetraacetic acid (Sequestrene Na 4, Alrose Chemical Co.) and autoclaved at 15 lb. pressure for 30 minutes. After autoclaving, glassware was rinsed 5 times with ion-free water obtained by passing distilled water through a Barnstead "Bantamac" demineralizer mode Bd-1. The effluent from the column contained less than .05 ppm total ionizable solids calculated as sodium ion equivalent. The 8-OH quinoline coprecipitation technic of Nicholas(5) was used to remove traces of iron and molybdenum from growth medium. This method was not suitable for investigation of calcium, so recrystallized analytical reagent chemicals were used. Nitrogen was determined by Kjeldahl semi-micro procedure(4). Turbidities were measured with Klett-Summerson colorimeter using the 660 mμ filter.

TABLE I. Effect of Nitrogen Source on Molybdenum Requirement of *Azotobacter vinelandii* O.

	μg N_2 fixed/ml*					
Exp.	N_2 .1 ppm Mo	N_2 −Mo	N_2 .2 ppm V	NH_4^+ .1 ppm Mo	NH_4^+ −Mo	Ashed NH_4^+
I				204	186	
II	205	50		201	200	
III		50			211	86
IV	·	56			211	
V	212	58		279	301	88
VI	262		83			
VII	166		62			

* In all tables, the ammonia grown cells harvested and washed with water, then resuspended in water and assayed for nitrogen. In all tables, "ashed NH_4^+" was prepared by heating ammonium acetate overnight in a porcelain crucible at 150°C. Residue was dissolved in dilute acid, neutralized and added to culture flasks.

Results. In agreement with previous studies with other strains, molybdenum and iron are both specifically required for nitrogen fixation process in *Azotobacter vinelandii* O. When ammonium ion is supplied as a nitrogen source the requirement is either eliminated, as with molybdenum or spared as with iron (Tables I and II). Vanadium has been reported to replace molybdenum in certain *Azotobacter* species(6) but this does not appear to be true with strain O.

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* Supported by grants from Rockefeller Foundation and Research Committee of Graduate School from funds of Wisconsin Alumni Research Foundation.

TABLE II. Effect of Nitrogen Source on Iron Requirement of *Azotobacter vinelandii* O.

	% maximum growth					
	N_2		NH_4^+		Ashed NH_4^+	
Added Fe^{+++} (ppm)	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
1.0	100	100	100	100		
.5	92	83	100	99		
.1	59	51	86	77		
0	17	14	16	14	16	10

Species of *Azotobacter* grown on N_2 possess an active hydrogenase capable of carrying out the knallgas reaction(7), and H_2 has been shown to be competitive inhibitor of the fixation of nitrogen(8). The effect of various levels of iron and molybdenum on hydrogen inhibition of nitrogen fixation by *Azotobacter vinelandii* O was studied. The effect was studied in a sealed system employing a KOH trap to remove respiratory carbon dioxide output. Any net gas uptake in the growth flask was replaced with pure oxygen. The flasks were equipped with colorimeter tube sidearms so that Klett-Summerson readings could be made without disturbing the gas phase. The results of a typical experiment with iron are shown in Fig. 1. As can be seen the growth constant is drastically reduced in presence of H_2, independent of the level of iron (1-10 ppm) in the medium. However, an increase in iron content of the medium caused a large increase in the apparent oxygen uptake of the culture. Sucrose utilization and CO_2 evolution values were not sufficient to account for the increase in oxygen uptake, indicating that excess gas uptake was a product of a stimulated knallgas reaction. The effect of molybdenum was also studied over a range of 0.001 to 1.0 ppm and always a large increase in apparent oxygen uptake was obtained when hydrogen was present. The amount of increased oxygen uptake, however, was the same for the entire range of molybdenum concentrations tested.

Uncertainty exists as to the requirement of calcium for nitrogen fixation. For example, Horner and Burk(9) demonstrated what appeared to be a specific requirement in *Azotobacter vinelandii* but later(10) stated that the requirement for calcium was unchanged whether atmospheric nitrogen or ammonium ion was used as a nitrogen source. Jensen(11) has reported that calcium is not required by *Azotobacter indicum* but is required by *Azotobacter chroococcum*. The results summarized in Table III demonstrate a specific calcium requirement for nitrogen fixation by *Azotobacter vinelandii* O. The presence of ammonium ion apparently eliminates the need for added calcium. Strontium can replace calcium at approximately the same molar concentration. During this study it was noted that levels of calcium lower than 5 μg per ml in a nitrogen free medium resulted in an ex-

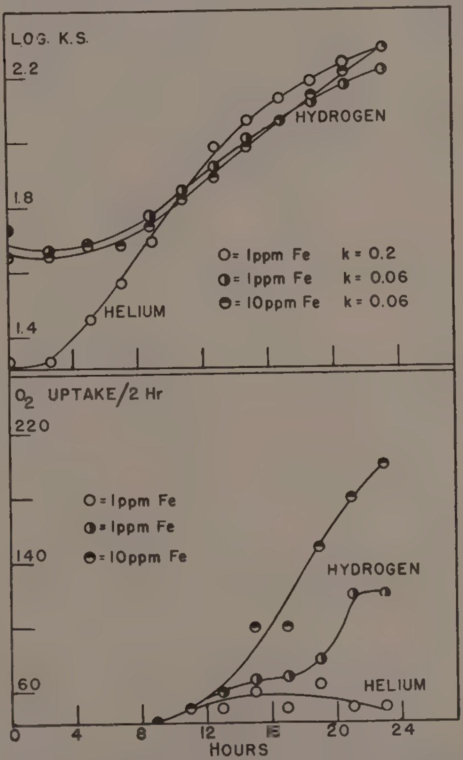

FIG. 1. Effect of iron conc. on hydrogen inhibition of nitrogen fixation by *Azotobacter vinelandii* O. k, velocity constant of growth =

$$\frac{2.3}{t_2 - t_1} \times \log \frac{KS_2}{KS_1}$$

in which KS_1 is Klett-Summerson reading at time t_1, etc. Gas phase contained 0.2 atm O_2, 0.2 atm N_2 and 0.6 atm H_2 or He. Oxygen uptake in ml/flask/2 hr.

TABLE III. Effect of Nitrogen Source on Calcium Requirement of *Azotobacter vinelandii* O.

Added Ca^{++} (ppm)	μg N_2 fixed/ml N_2	NH_4^+	Ashed NH_4^+
25	258	337	
10	241	308	
1	203	323	
.5	170	300	
0	13	313	26

tended lag period of growth followed by an apparently normal logarithmic phase. This is illustrated in Fig. 2. Varying the magnesium to calcium concentration ratio from 10 to 1 to 1 to 1 had no effect on the lag phase. There is an apparent interrelationship between calcium and phosphorus. Fig. 3 contains the results of an experiment demonstrating this interrelationship. Curves 1 and 2 show that phosphorus concentration does not effect the lag phase in the presence of an optimum amount of calcium. Curves 3 and 4 show that a low level of phosphorus results in a further lengthening of the lag phase of a culture with a suboptimal amount of calcium.

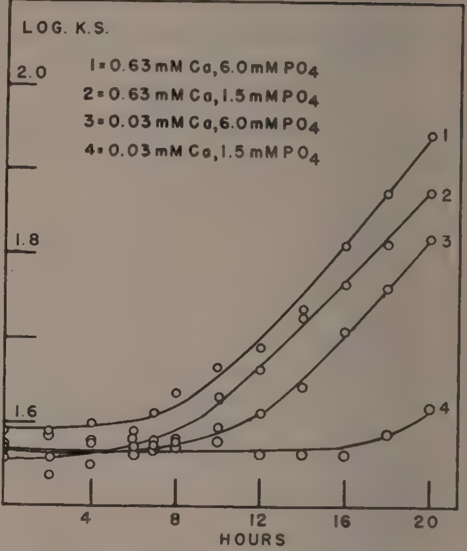

FIG. 3. Effect of varied calcium and phosphate conc. on lag phase of *A. vinelandii* O fixing atmospheric nitrogen.

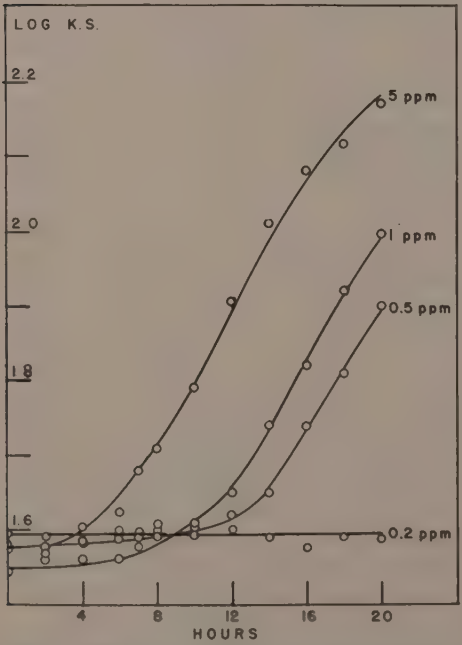

FIG. 2. Effect of calcium conc. (indicated ppm) on length of lag phase of *A. vinelandii* O fixing atmospheric nitrogen.

Discussion. The results of this study demonstrate that molybdenum, iron and calcium are specifically required for nitrogen fixation by *Azotobacter vinelandii* O, but the definite function of these metals in the fixation process is still to be established. However, molybdenum has been found to be a part of the hydrogenase enzyme system(12), and spectrophotometric studies of the effect of nitrogen and hydrogen on cell free preparations of nitrogen fixing organisms provide a function for iron via a hematin compound involved in the fixation process(13). The role of calcium in the system is even more obscure. The data reported here suggest that calcium functions in a reaction active during the lag phase, a reaction in which phosphate is also involved. The results with ammonium ion as a source of nitrogen indicate that the reaction or its products are not essential for the elimination of the lag phase unless the organism is actively fixing atmospheric nitrogen.

Summary. Molybdenum, iron and calcium ions have been shown to be specifically required when *Azotobacter vinelandii* O is fixing atmospheric nitrogen. Ammonium ion eliminates the need for molybdenum and calcium and has a sparing action on the requirement for iron. High levels of iron stimulate the knallgas reaction of *A. vinelandii* O fixing N_2 in the presence of H_2, but molybdenum in the range tested had no effect on this reaction. Low concentrations of calcium ions in a nitrogen-fixing culture result in an extended lag phase of growth. Decrease in the concentration of phosphate in the presence of low levels of calcium brings about a further increase in the lag, but this increase is not observed if the calcium supply is adequate.

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1. Burk, D., and Burris, R. H., *Ann. Rev. Biochem.*, 1941, v10, 587.

2. Jensen, H. L., *Bact. Revs.*, 1954, v18, 195.

3. Wilson, P. W., and Burris, R. H., *ibid.*, 1947, v11, 41.

4. Wilson, P. W., and Knight, S. G., *Experiments in Bacterial Physiology*, Burgess Publishing Co., Minneapolis, Minn., 1952.

5. Nicholas, D. J. D., *J. Hort. Sci.*, 1951, v26, 125.

6. Horner, C. K., Burk, D., and Allison, F. E., *J. Agr. Res.*, 1942, v65, 173.

7. Wilson, J. B., Lee, S. B., and Wilson, P. W., *J. Biol. Chem.*, 1942, v144, 265.

8. Wyss, O., and Wilson, P. W., *Proc. Nat. Acad. Sci., U. S.*, 1941, v27, 162.

9. Horner, C. K., and Burk, D., *J. Agr. Research*, 1943, v48, 981.

10. Burk, D., and Horner, C. K., *Proc. 3rd Intern. Congr. Microbiol.*, 1939, 489.

11. Jensen, H. L., *Proc. Linnean Soc. N.S. Wales*, 1948, v72, 299.

12. Shug, A. L., Wilson, P. W., Green, D. R., and Mahler, H. R., *J. Am. Chem. Soc.*, 1954, v26, 3355.

13. Shug, A. L., Hamilton, P. B., and Wilson, P. W., *Inorganic Nitrogen Metabolism.* Edited by B. Glass and W. D. McElroy, Johns Hopkins Press, Baltimore, Md., 1956, p344.

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Uricolysis in Normal and Leukemic Individuals. (22821)

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Hyperuricemia occurs with moderate frequency in leukemia and other blood dyscrasias. The hyperuricemia has been ascribed to increased nucleo-protein catabolism. In studies on the uric acid pool, Benedict(1) and her associates consistently found more urate produced than excreted. Using larger amounts of labeled uric acid, Wyngaarden (2) demonstrated that uricolysis occurs in significant amounts in humans. We attempted (3) to demonstrate the site of this uricolysis. The rate of urate disappearing from the formed elements of blood incubated with plasma was of a significant amount and could account for a large part of the degraded urate. Among our non-gouty hospitalized controls were several leukemics. These patients demonstrated a markedly lower rate of uricolysis than did the normals or even the gouty patients, the gouty patients being considerably lower than the normals. It is quite possible therefore, that elevated plasma urate levels in leukemics may be secondary, at least in part, to an impaired mechanism for uricolysis.

Methods. 50 ml of heparinized blood were obtained. A 5 ml sample was removed to serve as a whole blood specimen and the rest centrifuged. The plasma was removed and the buffy coat pipetted off. The buffy coat was centrifuged in a narrow tube in either the patient's own plasma or isotonic saline. After 3-4 separations, high concentrations of white blood cells relatively free of red blood cells were obtained. The red blood cell preparations were obtained by pipetting the red cells from the bottom of the tube and freed of white blood cells in the same manner as above. Each patient had the following samples incubated at 37° under 5% CO_2–95% O_2: 1. Whole blood. 2. Plasma. 3.White blood cells (0.5 ml) and the same patient's plasma (3. ml). 4. Red blood cells (0.5 ml) and the same patient's plasma (3. ml). Blood counts were done on preparations 3 and 4. On white cell preparations, the red counts

TABLE I. Mean Percentage Decrease in Plasma Urate after Incubation.

Subjects	No. of determinations	Mean plasma level	Whole blood	Plasma	White cells	Red cells
Normals	25	5.1 ± .1*	34.4 ± 2.3	2.3 ± 2.1	39.0 ± 2.2	29.8 ± 2.5
Leukemics	11	9.5 ± .2	22.4 ± 1.0	4.2 ± .3	21.4 ± .7	13.0 ± .7

* ± stand. error.

were less than 0.5 million, and in the red cell preparation, the white counts were less than 1000. For technical reasons, the patients chosen had increased leukocyte counts ranging from 18,000 to 542,000. Samples of plasma were withdrawn at 0, 4 and 24 hours of incubation and the plasma urate determined by the Yü modification of the Buchanan, Block and Christman method.

The results of 25 determinations on normal hospitalized patients as compared with 11 determinations on 7 leukemic patients are given in Table I.

To prove that the diminished rate of uricolysis was not related to the serum urate level, samples of normal sera were fortified by the addition of urate to hyperuricemic levels with no significant change in the rate of uricolysis. Of the leukemic patients, 3 had myelogenous leukemia, and 4 had lymphatic leukemia. There was no significant difference between the two types of leukemias we studied so far as diminished rates of uricolysis nor could differentiation be made as to the type of leukemia from the pattern of uricolysis. Aside from uricase, which has never been demonstrated in humans, 2 known enzyme systems are capable of oxidizing urate at a physiological pH. The first of these is peroxidase, which will oxidize urate in the presence of peroxide or a source of peroxide (as glucose and glucose oxidase) and the second is cytochrome-cytochrome oxidase(4). The end products of the peroxidase degradation were studied by Agner and by Tuttle and Cohen. The end products of cytochrome-cytochrome oxidase degradation have not been extensively studied.

Peroxidase is present to 1-2% of the dry weight of leukocytes of the myeloid series, while erythrocytes have both cytochrome and peroxidative activity(5). Agner states that leukocytes isolated from the blood in myeloid leukemia were very rich in peroxidase. The enzymatic activity of leukocytes in infections and in chronic myelogenous leukemia has been studied in regard to phosphatase content by Valentine(6). During a leukocytosis, the phosphatase activity may be as high as 5 times the normal and was found to be lower than normal in chronic myelogenous leukemia. Variable amounts of other enzymes are therefore also probable in disease states.

All urate determinations were total chromogen determinations and were not subjected to uricase degradation. Serious errors may still arise using a specific enzyme like uricase because of oxidation of other chromogenic substances or binding by the buffer of still other chromogenic substances(7). It is conceivable that uricolysis may proceed at a normal or even accelerated rate in leukemias but that substances reducing arsenophosphotungstic acid may be liberated into the plasma with the result that a defect in uricolysis is being demonstrated in the sample taken.

Summary. 1. Uricolysis occurs in whole blood as well as in plasma incubated with white or red blood cells. The rate of uricolysis is not dependent on the hyperuricemia in normals. There is marked defect in uricolysis in leukemics under the same experimental conditions. 2. Hyperuricemia in leukemia, may be in part due to diminution in the rate of uricolysis.

1. Benedict, J. D., Forsham, P. H., and Stetten, D., Jr., *J. Biol. Chem.*, 1949, v181, 183.
2. Wyngaarden, J. B., and Stetten, D., Jr., *ibid.*, 1953, v203, 9.
3. Bien, E. J., and Zucker, M., *Ann. Rheum. Dis.*, 1955, v14, 409.
4. Griffiths, M., *J. Biol. Chem.*, 1952, v197, 399.
5. Agner, K., *Acta Physiol. Scand.* 2 Suppl. 8, 1941.
6. Valentine, W. N., *Blood*, 1951, v6, 845.
7. Bien, E. J., and Troll, W., Proc. Soc. Exp. Biol. and Med., 1950, v73, 370.

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Antiglobulin Test in Anemia Caused by Acetylphenylhydrazine. (22822)

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Following intravenous injection of red cells from one individual to another of the same species, certain sensitizing antibodies may develop. They may be detected *in vitro* by the antiglobulin test (Coombs Test). This test depends upon the sensitization of red cells by such antibodies, with subsequent agglutination of these sensitized cells by antiglobulin serum. At times, autosensitization may occur, as in certain types of acquired hemolytic anemia. It has generally been concluded that, for unknown reasons, the affected individual developed antibodies to his own red cells, which were then destroyed, thus accounting for the anemia. On the other hand, it is possible that a positive Coombs test may be caused by injury of some other type, and may not involve a specific immunological reaction. This was suggested by a report of Muirhead, Groves and Bryan(1) who found a positive antiglobulin reaction in dogs during anemia produced by acetylphenylhydrazine. We have repeated the experiments, but failed, however, to confirm them. A preliminary search to account for the difference in results was unsuccessful so the disparity remains unexplained. Since these findings are of considerable theoretical importance, we feel the data should be recorded.

Methods. The technic of Muirhead, Groves and Bryan(1) was used, and, in addition, certain modifications were introduced in attempts to account for differences in results. Two different types of Coombs serum were prepared. The first (Serum A) was made by injecting whole canine serum into rabbits, exactly as directed(1). Since it seemed possible that the positive reactions reported(1) were due to antibodies other than antiglobulins, a second type of Coombs serum (Serum B) was prepared as follows: the globulin fraction was separated from canine plasma by ammonium sulfate precipitation. An alum precipitate was prepared from this(2). Rabbit antiserum was then produced and absorbed against pooled canine erythrocytes. The titers of the 2 types of Coombs sera were nearly the same, with identical specificities. On each day, both sera were used, and each was controlled by an indirect Coombs test against the test cells sensitized by a canine isoantibody. The dogs were given a single intravenous dose of 40 mg/kg of acetylphenylhydrazine as directed(1). Direct Coombs tests were performed at frequent intervals thereafter. In addition, in each instance, 2-fold serial dilutions of the Coombs sera were made in an attempt to demonstrate the prozone phenomenon reported(1). In doing these titrations some of the tests were set up using 1 ml of a 3% suspension of test erythrocytes rather than the 0.5 ml of a 2% suspension as was previously reported(1). Dogs 1-4 were done with the former, and dogs 5-8 with the latter. On several occasions both methods were used together and no difference was detected. The criteria for a positive reaction were those used by Muirhead, Groves and Bryan(1). Hematocrit determinations were done in Wintrobe tubes using heparinized venous blood.

Results. The results are summarized in the Table. Since all titrations were negative, only the result of the ordinary direct Coombs test are given. With the exception of one weakly positive test and 2 doubtful tests on the 9th day in dogs 1, 2 and 3, all were clearly negative. It may be of significance that these positive tests were all obtained against the Coombs serum produced against the whole canine serum. The tests using the true antiglobulin serum were uniformly negative. Whether the conflicting results of our experiments and those of Muirhead *et al.* were due to difference in the antiglobulin sera used remains undetermined. Further work along these and similar lines seems necessary.

Summary. No evidence of a positive antiglobulin test was found in dogs following injection of acetylphenylhydrazine. This is in

contradiction to the results of others.

TABLE I. Reactions of Canine Erythrocytes with 2 Types of Coombs Sera on Representative Days following a Single Intravenous Dose of Acetylphenylhydrazine.

Dog #	1			2			3			4		
Day after phenylhydrazine	Hct, %	Serum A*	Serum B†	Hct, %	Serum A	Serum B	Hct, %	Serum A	Serum B	Hct, %	Serum A	Serum B
0	49	—		45	—	—	56	—	—	51	—	—
1	41	—	—	34	—	—	51	—	—	44	—	—
4	31	—	—	26	—	—	40	—	—	36	—	—
7	27	—	—	28	—	—	27	—	—	28	—	—
9	30	±	—	31	±	—	34	1+	—	30	—	—
11–14	39	—	—	37	—	—	42	—	—	38	—	—
15–18	42	—	—	37	—	—	43	—	—	41	—	—
19–24	49	—	—	47	—	—	51	—	—	46	—	—

Dog #	5			6			7			8	
Day after phenylhydrazine	Hct, %	Serum A*	Serum B†	Hct, %	Serum A	Serum B	Hct, %	Serum A	Serum B	Hct, %	Serum B
0	46	—	—	41	—	—	53	—	—	44	—
1	37	—	—	36	—	—	42	—	—	23	—
4	33	—	—	23	—	—	31	—	—	29	—
7	31	—	—	30	—	—	27	—	—	26	—
9	33	—	—	30	—	—	27	—	—	25	—
11–14	36	—	—	33	—	—	35	—	—	34	—
15–18	38	—	—	35	—	—	39	—	—	34	—
19–24	46	—	—	46	—	—	44	—	—	42	—

* Serum A: Antiserum against whole dog serum.
† Serum B: " " dog globulin (see text).

1. Muirhead, E. E., Groves, M., and Bryan, S., *J. Clin. Invest.*, 1954, v33, 1700.

2. Kabat, E. A., and Mayer, M. M., *Experimental immunochemistry*, 1948, C. C. Thomas, Springfield, Ill., 543.

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Effect of Russell's Viper Venom (Stypven) on Stuart Clotting Defect. (22823)

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In a study of patient (R.S.) with congenital hemorrhagic disorder of moderate severity, Lewis, *et al.*(1) showed he had a prolonged one-stage prothrombin time which was corrected by normal plasma or serum but not by barium sulphate-adsorbed plasma or serum. The 2-stage prothrombin was normal and prothrombin consumption abnormal. These authors believed that the factor deficient in this patient was identical to that previously described by Alexander, *et al.*(2) Koller, *et al.*(3) and Owren(4) and now referred to as SPCA, factor VII or proconvertin (the 3 terms being used synonymously). It has now been established that factor VII (SPCA or proconvertin) is not essential for generation of blood thromboplastin(5,6). In a re-study of patient (R.S.) it has been found that the patient's serum is deficient in a factor essential for blood thromboplastin formation(7). Thus if various concentrations of normal serum in the patient's serum are prepared and incubated with normal $Al(OH)_3$-adsorbed plasma and platelets in the thromboplastin generation test, maximum yield of thromboplastin generated is proportional to amount of normal serum in the mixture. The factor deficient in this patient therefore differs from factor VII. However the two factors appear to have many properties in common, both being relatively heat stable, present in serum as well as plasma and necessary for a normal one-stage prothrombin time(7).

Since Russell's viper venom (Stypven) completely corrects the prolonged one-stage prothrombin time of plasma deficient in factor VII(8,9) it appeared reasonable to determine whether Stypven would also correct the clotting defect of the patient (R.S.)

Materials and methods. Whole blood was collected in glass tubes, and allowed to clot and stand at 28°C for 24 hours before separating the serum. Plasma was obtained by adding 9 parts whole blood to 1 part 3.8% trisodium citrate and centrifuging at 3,000 rpm. Russell's viper venom (Stypven), obtained from Burroughs Wellcome and Co., was diluted with distilled water. Platelet suspensions were prepared from a normal subject by the technic used in the thromboplastin generation test(10). A 0.1% suspension of crude cephalin(11) in distilled water was used. Modified one-stage prothrombin times were determined by mixing 0.1 ml 0.1% cephalin suspension, 0.1 ml platelet suspension or fresh thromboplastin solution (Difco), 0.1 ml Stypven solution, 0.1 ml plasma and 0.1 ml 0.025M $CaCl_2$ in the order named.

Results. Effect of varying concentrations of Stypven on modified one-stage prothrombin time of patient's plasma. Various concentrations of Stypven were prepared ranging from 1 in 1,000 to 1 in 100,000 and the modified one-stage prothrombin time of normal or patient's (R.S.) plasma determined using Difco brain thromboplastin and each dilution of Stypven (Table I). It is to be noted that varying the concentration of Stypven did not result in any significant correction of the patient's prothrombin time, which remained between 2½ and 3 times that of normal control for the particular dilution of Stypven under test. Similar results were obtained if either a platelet suspension or a 0.1% suspension of crude cephalin was substituted for the brain thromboplastin used in the above experiment.

TABLE I. Effect of Concentration of Stypven on Modified One-Stage Prothrombin Time Using Difco Rabbit Brain Thromboplastin.

.1 ml plasma, .1 ml Difco brain thromboplastin, .1 ml of Stypven in conc. listed below, .1 ml 0.025M $CaCl_2$,	Clotting times, sec.	
	Normal plasma	Patient's plasma
1 in 1,000	8.9	23.5
2,500	5.5	15
5,000	5	14.6
10,000	6.6	18.8
20,000	8.5	22
50,000	11	27
100,000	11.8	35
Saline control	13.8	42

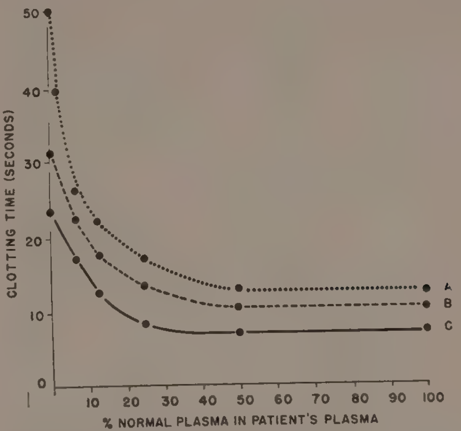

FIG. 1. Dilution curves obtained by determining one-stage "prothrombin" times of various dilutions of normal plasma in patient's (R.S.) plasma using, as sources of thromboplastin, a mixture of brain thromboplastin and (A) saline, (B) "Stypven" diluted 1 in 60,000 and (C) "Stypven" diluted 1 in 10,000.

Effect of diluting patient's plasma with normal plasma. Serial dilutions of patient's plasma in normal plasma were prepared and the modified one-stage prothrombin times of each dilution determined using Difco brain thromboplastin with either Stypven or saline (Fig. 1). Two concentrations of Stypven were used, 1 in 10,000 which was found to be the optimal dilution for the batch of Stypven used in this experiment and a 1 in 60,000 dilution. The result of such an experiment is shown in Fig. 1, where it can be seen that the

3 curves are roughly parallel: the one-stage prothrombin time and modified one-stage prothrombin, increasing in the same proportion when the concentration of normal plasma in the patient's plasma is diminished.

Discussion. It has already been shown that the patient (R.S.) is not deficient in either PTC or prothrombin and that mixtures of plasma from a case of congenital deficiency of SPCA (factor VII) and the patient's plasma are mutually corrective(7). The factor deficient in the patient differs from factor X since this factor affects the yield but not the rate of blood thromboplastin formation. The factor, deficient in the patient, also differs from AHF and factor V which are not present in normal serum and from the Hageman factor(12), PTA(13) and the fourth thromboplastin factor(14) since deficiencies of these factors do not affect the one-stage prothrombin time.

Telper, *et al.*(15) have recently described a patient with a congenital hemorrhagic diathesis due to deficiency of a factor which resembles factor VII but is necessary for blood thromboplastin formation. This factor, which is referred to as the Prower factor, resembles that deficient in our patient. The clotting defect is, however, completely corrected by Stypven while the present results show clearly that the clotting defect in the patient (R.S.) is not corrected by Stypven. This difference cannot be explained on a technical basis as one of the technics used in the present work was the same as that employed by Telper, *et al.*, and similar results were obtained using modifications of this technic. Moreover the difference is not merely a quantitative one for the patient of Telper, *et al.* had a one-stage prothrombin time which was more than twice the normal control indicating that the clotting deficiency was a moderately severe one. The two clotting defects must therefore be distinct and the factor deficient in the patient (R.S.) is referred to as the Stuart factor after the patient's surname.

The possibility that the patient (R.S.) is deficient in two factors has to be considered. A multiple congenital defect would imply that the patient was deficient in the Stuart factor and one or another *new* clotting factor including possibly the Prower factor since a deficiency of all the other known coagulation factors including Christmas factor (PTC) has been excluded. Since 2 other unrelated patients suffering from congenital hemorrhagic diatheses have been studied and found to have identical clotting defects(1,7), the multiple deficiency hypothesis is highly unlikely. The probabilities of the same double homozygosity or homozygosity at one locus, and heterozygosity at the other occurring independently 3 times in a small population are exceedingly small. The possibility of a multiple congenital deficiency can therefore be discarded with a reasonable degree of safety.

The finding that Stypven does not correct the Stuart defect however, does more than establish its separate identity. It is of practical importance since a simple prothrombin assay method has been described in which it is assumed that the one-stage "prothrombin time" is a true measure of prothrombin when Stypven is added to the brain thromboplastin (16). Such an assay procedure however is a function of both prothrombin and Stuart factor. It has been pointed out elsewhere(7) that the usual assay procedures for factor VII (proconvertin) as described by Owren(17) and Koller *et al.*(3) measure both Stuart factor and factor VII. These asbestos filtered plasmas retain considerable prothrombin but are deficient not only in factor VII (proconvertin) as these workers have assumed but Stuart factor and probably Prower factor. However, the simple addition of Stypven to such substrate plasmas should allow these assay technics to measure specifically the Stuart factor.*

Summary. It is shown that Russell's viper venom (Stypven) does not correct the prolonged one-stage prothrombin time of patient with a congenital hemorrhagic diathesis previously thought to be deficient in factor VII. The factor deficient in this patient which is referred to as the Stuart factor, is readily distinguishable from both the Prower factor and factor VII since the prolonged prothrombin times of plasma deficient in either of these

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* To be published.

two factors are completely corrected by Stypven. The finding that Stypven does not correct the clotting defect of Stuart factor deficient plasma implies that certain modified one-stage "specific" assay methods for prothrombin using Stypven as a source of factor VII are not, in fact, specific, and measure both prothrombin and Stuart factor.

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1. Lewis, J. H., Fresh, J. W., and Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1953, v84, 651.

2. Alexander, B., Goldstein, R., Landwehr, G., and Cook, C. D., *J. Clin. Invest.*, 1951, v30, 596.

3. Koller, F., Loeliger, A., and Duckert, F., *Acta Hemat.*, 1951, v6, 1.

4. Owren, P. A., *Scand. J. Clin. and Lab. Invest.*, 1951, v3, 168.

5. Duckert, F., Fluckiger, P., Matter, M., and Koller, F., PROC. SOC. EXP. BIOL. AND MED., 1955, v90, 17.

6. Hicks, N. D., *Med. J. Aust.*, 1955, v2, 331.

7. Graham, J. B., and Hougie, C., *Proc. 6th Internat. Conf. Hemat.*

8. Jenkins, J. S., *J. Clin. Path.*, 1954, v7, 287.

9. Rapaport, S. I., Aas, K., and Owren, P. A., *Blood*, 1954, v9, 1185.

10. Biggs, R., and Douglas, A. S., *J. Clin. Path.*, 1953, v6, 23.

11. Bell, W. N., and Alton, H. G., *Nature*, 1954, v174, 880.

12. Ratnoff, O. D., and Colopy, J. E., *J. Clin. Invest.*, 1955, v34, 602.

13. Rosenthal, R. L., *Am. J. Med.*, 1954, v17, 57.

14. Spaet, T. H., Aggeler, P. M., and Kinsell, B. G., *J. Clin. Invest.*, 1954, v33, 1095.

15. Telfer, T. P., Denson, K. W., and Wright, D R., *Brit. J. Haemat.*, 1956, v2, 308.

16. Hjort, P., Rapaport, S. I., and Owren, P. A., *J. Lab. Clin. Med.*, 1955, v46, 89.

17. Owren, P. A., and Aas, K., *Scand. J. Clin. and Lab. Invest.*, 1951, v3, 201.

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Oxygen Uptake and Lactate Formation of HeLa Cells.*† (22824)

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HeLa cells were obtained from an epidermoid carcinoma of the cervix and started as a tissue culture in 1951(5). After 4 years *in vitro*, smears stained by Papanicolou's technic (4) indicated that HeLa had malignant features. It has been grown by Toolan in cortisone-treated rats and in human subjects in terminal stages of another neoplastic process (5). The cells are uniform in appearance as compared to mixed cell types found in tissue. They can be easily separated without apparent injury. Cell number or weight can be determined quite accurately since there is little interstitial material. For these reasons HeLa makes an ideal cell suspension on which to study metabolism. Our primary objective was to compare metabolism of human HeLa cells to that reported characteristic of animal tumors(2). Originally Gey started HeLa in medium which contained human serum. Other laboratories§ adapted a line of HeLa cells to grow in medium containing horse serum. Our second objective was to compare metabolism of cells cultured in human serum‖ with that of cells cultured in horse serum.

Procedure. At start of this study 2 lines of HeLa were carried in medium containing 40% Earle solution, 20% chick embryo extract (50:50) and 40% horse or human serum. It was desired to carry one line of cells

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[‡] Part-time Medical Student Research Fellow, U.S.P.H.S.

[§] Original cultures obtained from Dr. W. R. Earle.

[‖] Serum furnished by Amer. Red Cross (Dr. R. M. Joyer) and Corn State Laboratories (Dr. N. K. Jarvis).

in human medium. Subcultures of each line were placed in semi-synthetic medium which had proven adequate for indefinite growth of Earle's Strain L cells. An initial solution contained 0.25 g yeast¶ and 0.25 g lactalbumin hydrolysates/liter Earle solution. Horse or human serum (10%) was added before use. Culture medium was changed 3 times weekly and all metabolic determinations made the day following a culture change. Determinations on HeLa were made in 2 suspending solutions. In one, phosphate solution alone was used, in the other 10% neutralized horse or human serum(10) was added to it. Respiration was determined by direct method of Warburg(9) which does not allow use of Earle solution because of its high bicarbonate content. Phosphate buffered solution (pH 7.4) adopted for use, contained the following materials per liter: 8.12 g. NaCl, 0.28 KCl, 0.15 $CaCl_2$, 0.33 $MgSO_4 \cdot 7 H_2O$, 0.28 Na_2HPO_4, 0.05 $NaH_2PO_4 \cdot H_2O$, 1 Glucose, 0.25 lactalbumin hydrolysate, 0.25 yeast hydrolysate. Lactate was determined chemically by the method of Barker and Summerson(1). Concentration in one reaction vessel was obtained immediately after equilibration for temperature and in another an hour later. Difference between concentrations in the 2 vessels was taken as lactate formation. During experimentation, number of HeLa cells in a reaction vessel was determined by counting on a hemacytometer. To obtain dry weight intercellular material was digested with 0.25% Difco trypsin (1:250) in Earle solution. The cells were collected by centrifugation, resuspended in Earle solution, counted, then collected again and dried at 110°C to constant weight. Three determinations indicated that one million HeLa cells weighed 0.99 mg. Phenylenediamine was used to obtain a measure of functional cytochrome reserve. This was done by adding p-phenylenediamine from side arm of reaction vessel so that final concentration was 0.02 M. Respiration was then compared with that in control flask without p-phenylenediamine. In preliminary respiration experiments, 2 methods of separating cells were tried. Trypsin was used in one method. In the other, cells were brushed from floor of T-60 flasks with brush made of stainless steel and nylon bristles and separated further by filling and discharging them through a pipette 15 times. Since trypsin appeared to depress respiration in some cases (Table I), use of nylon brush was considered the better method for cell separation. It is necessary to assume a constant rate of oxygen uptake for a definite interval to express Q_{O_2} values as mm^3/hr. Rate of oxygen uptake for practical purposes was constant for one hour, but slightly less at 2 hours (Fig. 1). Subsequent determinations were run for exactly one hour. Respiration was higher at 38°C than at 37.5°C ($P < .01$) but the lower temperature was used for metabolic determinations because incubators used for growing cultures had been maintained at 37.5°C.

TABLE I. Effect of Separation Methods on Respiration of HeLa.

Method	Culture serum	Warburg sol.	Temp., °C	Q_{O_2}	P
Brush	Horse	Saline	37.5	2.2 (8)	
Trypsin			37.5	2.2 (5)	
Brush	Horse	Serum	38.0	7.7 (2)	$>.05$
Trypsin			38.0	5.8(13)	
Brush	Human	Saline	37.5	8.4 (6)	$<.05$
Trypsin			37.5	4.7 (5)	

Figures in parentheses indicate No. of determinations.

Results. Comparison was made either between quotients obtained in saline or between those in saline-serum. Without exception, all metabolic quotients were higher for HeLa cells grown in medium containing human se-

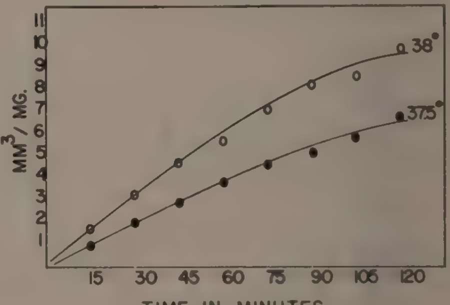

FIG. 1. Oxygen uptake of HeLa in neutralized horse serum.

¶ Nutritional Biochemicals Corp.

TABLE II. Respiration and Glycolysis at 37.5°C of HeLa Grown in Media Containing Either Human or Horse Serum.

Culture serum	Warburg solution	Q_{O_2}	$Q^{O_2}_{LA}$	$Q^{N_2}_{LA}$	P-PD
Horse	Saline	2.2 (13)	6.7 (3)	3.2 (2)	11.9 (3)
Human	"	6.3 (11)	10.2 (12)	8.4 (6)	14.3 (5)
Horse	" + horse serum	4.0 (13)	7.2 (4)	5.2 (5)	21.7 (4)
Human	" + human "	6.0 (4)	12.0 (4)	5.5 (5)	26.3 (4)

All values expressed as mm³/mg dry wt/hr. Figures in parentheses indicate No. of determinations. P-PD = Q_{O_2} when 0.02 M p-phenylenediamine added.

rum than those grown in a horse serum medium (Table II).

Quotients determined in saline and in serum were about the same order of magnitude. There were exceptions. Respiration of HeLa adapted to grow in horse serum, when determined in saline, was lower than similar determinations in horse serum ($P < .05$). Phenylenediamine stimulated respiration of cells in solution containing serum to a greater extent than it did when saline alone was used; this was true for cultures which had been grown in horse serum ($P < .01$) or human serum ($P < .05$).

It has been pointed out by Warburg, Burk and others(2,9) that tumors have a metabolism that differs from normal tissues. HeLa cells, carried by tissue culture technic, metabolized in some ways like *in vivo* derived tumor slices but in other ways like normal tissue(6) (Table II). Metabolism of HeLa cells was similar to that reported for tumors, in that respiration (Q_{O_2}) was moderately low and aerobic lactic acid formation ($Q^{O_2}_{LA}$) moderately high. They exhibit metabolism dissimilar to that reported for tumors in that anerobic lactic acid formation ($Q^{N_2}_{LA}$) was low and added p-phenylenediamine stimulated respiration greatly (338%). The latter observation was indirect evidence that adequate functional cytochrome reserve was present. There was no Pasteur Effect ($Q^{N_2}_{LA} - Q^{O_2}_{LA}$) because anerobic glycolysis was not in excess of aerobic glycolysis. Respiration and anerobic glycolysis were about the same magnitude and thus Fermentation Excess ($Q^{N_2}_{LA} - 2Q_{O_2}$) was negative.

Discussion. Numerous workers have shown that animal cells grown in chemically defined medium need, among other things, certain amino acids and vitamins. In an attempt to obtain a simple homologous medium enzymatic lactalbumin hydrolysate was used because Melnick and Riordon(8) had found it to be growth stimulating; enzymatic yeast hydrolysate was used so that most B vitamins as well as amino acids would be present. Hydrolysates were added to the medium in concentration that would yield an amino acid solution very similar to that found in serum (7). The medium was sterilized by Selas filtration rather than by autoclaving(8) so that thiamin, folic acid and vit. B_{12} would not be destroyed. Preliminary results indicated that hydrolysate culture medium, as used in this study, compared favorably to Eagle's synthetic medium(3) supplemented with 10% serum.

One early observation which we did not readily accept was that aerobic glycolysis was higher than anerobic glycolysis even when bound bicarbonate was present in neutralized serum. Our repeat experiments gave similar results. Warburg(9) has shown that glycolysis of more than 20 different tumors was higher under anerobic than aerobic conditions. It is interesting to note that Hellerman collaborating with Gey(5) found that aerobic lactate was higher than anerobic lactate for the 14P rat fibroblast cultured *in vitro*.

Summary. Gey's strain HeLa cells were cultured in Earle T-60 flasks for almost a year in media composed of 0.25 g. yeast and 0.25 g. lactalbumin hydrolysate/l Earle solution with 10% human or horse serum. Respiration was determined manometrically and lactate formation chemically. In preliminary experiments, 0.25% Difco trypsin (1:250) when used to separate cells, depressed respiration in some instances. Respiration of sus-

pensions of HeLa was constant for an hour but decreased slightly after first hour. Metabolism of two lines of HeLa cells was compared. Respiration and glycolysis of cells grown in media containing homologous human serum was higher than that of cells grown in media containing horse serum. Human HeLa cells had a metabolism like that reported for tumor tissue in that respiration was low ($Q_{O_2} = 6.0$) and aerobic glycolysis moderate ($Q_{LA}^{O_2} = 12.0$). They differed from tumor tissue in that anerobic glycolysis was low ($Q_{LA}^{N_2} = 5.5$) and p-phenylenediamine stimulated respiration 338% ($Q_{O_2} = 26.3$). There was no Pasteur Effect and Fermentation Excess was negative.

1. Barker, J. B., and Summerson, W. H., *J. Biol. Chem.*, 1941, v138, 535.

2. Burk, D., Earle, W. R., Winzler, R. J., *J. Cancer Inst.*, 1944, v4, 363.

3. Eagle, H., *Science*, 1955, v122, 501.

4. Fennell, R. H., *Anat. Rec.*, 1956, v124, 10.

5. Gey, G. O., *The Harvey Lectures*, New York, Academic Press, 1954-55, Series L, 154.

6. Greenstein, J. P., *Biochemistry of Cancer*, New York, Academic Press, 1954, 451.

7. Krebs, H. A., *Ann. Rev. Biochem.*, 1950, v19, 409.

8. Melnick, J. L., and Riordon, J. T., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 208.

9. Warburg, O., *The Metabolism of Tumors*, London, Constable and Co., 1930.

10. Warren, C. O., *J. Biol. Chem.*, 1944, v156, 559.

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Absence of Appreciable Resistance to Growth Inhibiting Action of Cortisone After Prolonged Treatment.* (22825)

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Chronic administration of nitrogen retaining hormones, to the plateaued adult Norway rat, leads to period of rapid growth followed by attainment of a new weight plateau on which there is no further growth although dosage of hormone per animal or per unit weight, is unchanged. This phenomenon of plateauing on chronic treatment with anabolic hormones appears to be identical in protein anabolic steroids(1,2) in anterior pituitary growth hormone(3-5); and in anabolic steroids plus growth hormone(6). Glucocorticoids have been shown to have a striking growth inhibiting action(7,8) due to their protein catabolic action. It would, therefore, be of interest to determine if there is a failure of constant dosage of glucocorticoid to give a constant inhibition of growth; indeed, the data of Tolksdorf *et al.*(8) suggest that this might be the case.

The present investigation was performed to further clarify possible development of resistance to growth inhibiting action of glucocorticoids. Since the time required for growth cessation, on a constant dosage of anterior pituitary growth hormone, is highly sensitive to the dose level(9), a range of dose levels was employed.

Methods. A microcrystalline suspension of cortisone acetate† (Δ^4-Pregnene-17β, 21-diol-3,11,20-trione-21-acetate) in isotonic saline was prepared by trituration with Tween-80 (polyoxyethylene sorbitan monooleate). Concentration of the suspension was adjusted to contain 3.2 mg cortisone/ml and less than 0.2 % Tween-80. After dilution, the suspension was sealed in injection vials and stored at 2 to 4°C until just before injection. Single daily subcutaneous injections were given under strictly aseptic conditions, at systematically varied sites along dorsal surface of the rat. Dosage was adjusted every 2 days

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† Cortisone used was generously contributed by Dr. C. J. O'Donovan and Department of Clinical Investigation, The Upjohn Co., Kalamazoo, Mich.

TABLE I. Weight Gain of Cortisone Treated Young Female Rats, in Successive 10 Day Intervals, before and during Treatment.

Cortisone dose, mg/kg/day	No. of rats	Wt gain (g)						Mean wt (g) 1st day of treatment
		10 days pretreat.	10 day intervals of treatment					
			1st	2nd	3rd	4th	5th	
0	15	40.9 ± 2.0*	35.3 ± 1.7	27.0 ± 1.6	19.2 ± 1.3	12.1 ± 0.9	12.3 ± 1.1	98.4
8	8	38.3 ± 2.8	17.0 ± 2.7	17.0 ± 2.3	14.5 ± 2.1	6.7 ± 1.8	5.2 ± 1.4	99.0
16	8	40.6 ± 2.6	5.7 ± 2.2	12.1 ± 2.8	10.8 ± 1.0	6.8 ± 1.7	6.2 ± 1.5	98.5
24	8	41.6 ± 3.1	–2.6 ± 2.8	8.9 ± 1.4	10.6 ± 1.6	3.1 ± 1.6	2.2 ± 2.2	99.6
32	8	41.9 ± 2.4	–4.1 ± 2.0	4.4 ± 2.1	6.1 ± 2.0	8.0 ± 2.1	3.1 ± 1.1	99.9

* Mean ± stand. error of mean in all cases.

to maintain dosage/kg body wt constant. The rats were rewarded with 0.5 ml imitation maple syrup after each injection. Split litter controls were given the same amount of syrup, but were not given injections of the suspending medium since previous work in this laboratory has shown 1 cc/day of 0.2% Tween-80 to be devoid of effect on growth. In experiments reported on anabolic hormones, stanolone (Androstane-17β-ol-3-one) was suspended in saline with Tween-80 in the same manner as cortisone and adjusted to concentration of 10 mg stanolone/ml. Ant. pituitary growth hormone was prepared in this laboratory by modification of the method of Wilhelmi, Fishman and Russell(10). The growth hormone was dissolved in sodium carbonate-saline at pH 9.5; adjusted to growth hormone concentration of 2 mg/ml; divided into injection vials and stored frozen at –30°C until just prior to injection. All experiments were carried out in the room in which rats had been raised from birth. Temperature was maintained at 22 to 24°C and all groups were fed Purina Laboratory Chow *ad lib*. The rats were an all black strain, developed in this laboratory from original Long-Evans stock. Rats on the cortisone experiment were young females with mean age of 38 days, and mean wt of approximately 100 g on the day treatment was initiated. Rats on the anabolic hormone experiments were plateaued young adult females, 8-10 months of age, having a mean wt of 250 g at onset of treatment.

Results. Table I gives growth of young female rats on cortisone treatment during 10 days prior to treatment and during 5 successive 10 day periods of treatment. The decrease in growth rate of control animals during each successive 10 day interval is the characteristic plateauing as adult size is approached. This is probably also the explanation of the successive decrease in growth in the group treated with 8 mg/kg/day. A complete cessation of growth was achieved only at the 24 mg/kg/day and 32 mg/kg/day dose levels, and then only during the first 10 day period. There appears to be no meaningful increase in growth in any group after the second 10 day period on treatment. Furthermore, during the final 10 day period, neither the 24 mg/kg/day, nor the 32 mg/kg/day group achieved a growth equal to 10% of that shown by normal controls when at the same body wt.

In comparison, Table II shows the typical decrease in resumed growth of adult animals given growth promoting hormones over intervals corresponding to those used in the cortisone experiments.

Discussion. We interpret the data presented above to indicate that no appreciable loss in growth inhibiting action of glucocorticoids occurs, at any dose level utilized, within a time interval comparable to that required for cessation of the resumed growth of adult rats treated with growth promoting hormones.

Summary. Cortisone acetate was given daily at 4 dose levels, for 50 days, to young female Long-Evans rats, initially 38 days of age and weighing approximately 100 g each. The upper 2 dose levels were in the range giving nearly complete cessation of growth. In the upper 2 dose levels, there appeared to be a slight reduction in growth inhibiting action after the first 10 days, but all 4 groups still had a marked depression of growth during the 40th to 50th day of treatment. For comparison, anterior pituitary growth hor-

TABLE II. Weight Gain of Plateaued Adult Female Rats Treated with Pituitary Growth Hormone and Stanolone, in Successive 10 Day Intervals before and during Treatment.

		Wt gain (g)					
			10 day intervals of treatment				
Treatment and dose	No. of rats	10 days pretreat.	1st	2nd	3rd	4th	5th
Growth hormone, 1 mg/rat/day	8	0.9 ± 2.0*	38.6 ± 2.8	21.8 ± 4.6	−5.8 ± 5.7	−3.0 ± 3.1	−1.6 ± 1.0
Growth hormone, controls	20	0.7 ± 0.7	4.9 ± 1.3	2.8 ± 1.1	−1.2 ± 1.3	2.9 ± 1.1	−2.4 ± 1.0
Stanolone, 5 mg/rat/day	6	1.2 ± 1.6	19.3 ± 2.3	6.3 ± 1.3	6.0 ± 0.5	3.7 ± 1.6	
Stanolone, controls	18	3.1 ± 0.8	2.3 ± 0.7	0.0 ± 1.4	5.1 ± 1.1	2.0 ± 1.2	

* Mean ± stand. error in all cases.

mone and stanolone were given to plateaued adult female rats of the same strain. These hormones, at dose levels capable of giving a major growth response during the first 20 days of treatment, gave little response after the first 20 days.

1. Kochakian, C. D., *Am. J. Physiol.*, 1950, v160, 53.

2. Kochakian, C. D., and Beal, B., *ibid.*, 1950, v160, 62.

3. Selye, H., *Ann. d'Endocrinol.*, 1952, v13, 841.

4. Emerson, J. D., *Am. J. Physiol.*, 1955, v181, 390.

5. Emerson, J. D., and Emerson, G. M., *ibid.*, 1955, v182, 521.

6. Selye, H., *First Annual Report on Stress*, Acta Inc., Montreal, 1951, 237.

7. Selye, H., *Am. J. Physiol.*, 1952, v171, 381.

8. Tolksdorf, S., Battin, M. L., Cassidy, J. W., McLeod, R. W., Warren, F. H., and Perlman, P. L., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 207.

9. Emerson, J. D., and Emerson, G. M., *Am. J. Physiol.*, 1955, v183, 612.

10. Wilhelmi, A. E., Fishman, J. B., and Russell, J. A., *J. Biol. Chem.*, 1948, v176, 735.

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Serum Protein-Bound Carbohydrates and Lipids in Experimental Tuberculosis. (22826)

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Guinea pigs inoculated with tubercle bacilli reveal characteristic serum protein changes which resemble to a great degree those in human tuberculosis; serum gamma globulin is elevated and albumin reduced(1,2). These serum protein changes, especially the gamma globulin elevation, have been related to chronic inflammation and hypersensitivity reactions. However, similar changes occur in chronic hepatic disease. It appeared therefore desirable to further characterize the serum protein changes produced by chronic tuberculosis.

A rise of serum mucoproteins (which are alpha globulins) (3) in experimental tuberculosis(4), in human tuberculosis(5) and in experimental brucellosis(6) has been demonstrated. This rise is absent in hepatic injury (7). Since methods have become available recently to demonstrate serum protein-bound lipids(8) and carbohydrates(9) by zone electrophoresis it appeared worthwhile to study their levels in experimental tuberculosis. To accentuate the serum protein changes, animals with long standing tuberculosis were investigated. The levels were compared not only with those of healthy control guinea pigs but also with animals in which treatment with isoniazid temporarily prevented development of tuberculosis(10).

Materials and methods. Serum was obtained from 12 guinea pigs inoculated subcutaneously into the right inguinal region with 0.1 mg of tubercle bacilli, strain H37Rv, and exhibiting far advanced tuberculosis at autopsy. Another group of 7 animals similarly infected received 20 mg isoniazid daily for 2 months following inoculation and were free of tuberculosis grossly and microscopically at autopsy. All animals were sacrificed 8 to 16 months after inoculation simultaneously with 12 control guinea pigs kept for the same period and free of any tuberculosis at autopsy. The following were determined in the serum: total proteins by micro-Kjeldahl and Nesslerization; gamma globulin turbidimetrically according to Huerga and Popper (11); mucoproteins also turbidimetrically according to Huerga *et al.*(12); hexosamines according to Elson and Morgan(13); protein-bound hexoses according to Lustig and Langer(14) and total lipids turbidimetrically according to Huerga *et al.*(15). In addition 3 paper strips, on which serum had been placed, were exposed to vertical zone electrophoresis. One was subsequently stained with bromphenol blue for proteins(16), another with periodic acid leucofuchsin (Schiff reaction) for protein-bound carbohydrates(9) and a third one with Oil Red O, for protein-bound lipids according to Durrum(8). The relative amount of protein as well as protein-bound carbohydrates and lipids were determined by integrating photometric scanner (Analytrol). The amounts found were expressed in percentage, and absolute amounts were calculated using as reference for proteins the total protein; for protein-bound carbohydrates the sum of hexosamines and protein-bound hexoses; and for protein-bound lipids the total lipids. In addition lipid and carbohydrate content of each serum protein fraction was calculated and expressed in percent of that fraction.

Results. In guinea pigs with active tuberculosis serum albumin was moderately reduced and gamma globulin and mucoprotein were significantly elevated. These changes, as well as those to be reported below, were absent in inoculated animals in which isoniazid treatment prevented active tuberculosis.

Total serum lipids as well as lipoprotein fractions were not influenced by tuberculosis. Both total serum hexose and hexosamine were slightly elevated in active tuberculosis as were all protein-bound carbohydrates, if expressed in absolute amounts. However this increase was not statistically significant and the relative distribution of carbohydrates was not significantly altered. Carbohydrate concentration of gamma globulin however, was distinctly depressed in guinea pigs with tuberculosis while that of alpha globulin and beta globulin were not significantly altered.

The very small amount of carbohydrates in the large fraction of albumin discouraged calculation of this figure.

The failure of the carbohydrate moiety of the gamma globulin to rise parallel with protein, resulted in a lower carbohydrate concentration in this protein fraction. This reflected itself in the ratio of carbohydrate concentrations, (carbohydrate percent in alpha globulin/carbohydrate percent in gamma globulin) and in the alpha globulin/gamma globulin ratio of the proteins (Table I). The former increased, while the latter decreased in tuberculous animals.

There is an increase in mucoproteins in tuberculous animals.

Discussion. Since the most significant alteration in protein fractions in experimental guinea pig tuberculosis involves albumin and gamma globulin, it is not surprising that lipoproteins, mainly components of alpha and beta globulins, are not significantly altered in these conditions. The slight rise of alpha globulin under these circumstances is reflected in marked elevation of mucoproteins which are alpha globulins(3). Since mucoproteins represent only a very small fraction of alpha globulin their marked rise which also runs parallel with elevation of total carbohydrates, is not sufficient to produce significant changes of serum concentration of the alpha globulin. The relatively small rise is associated with a parallel rise of alpha globulin-bound carbohydrate which suggests that the excess amount of alpha globulin does not differ in carbohydrate content. However, this rela-

TABLE I. Serum Protein Fractions in Control and Tuberculous Guinea Pigs.

	Controls		Tuberculous active			Tuberculous successfully treated		
	Mean	SEm	Mean	SEm	$\frac{OD}{SE\text{-diff.}}$	Mean	SEm	$\frac{OD}{SE\text{-diff.}}$
No. of animals	14		12			7		
Albumin, g %	3.1	.13	2.6	.03	3.6	3.1	.10	.0
α-globulin, ”	1.6	.13	1.9	.04	2.1	1.7	.04	.7
β-globulin, ”	.9	.04	.8	.05	1.5	.8	.06	.0
γ-globulin, ”	.6	.05	1.6	.07	40.0	.8	.10	1.8
Mucoproteins, mg %	323	21	537	24	6.5	346	15	.9
Total lipids, ”	217	6.2	201	16	.9	191	17	1.4
α-lipoproteins, ”	128	7.0	131	17.7	.1	130	17	.1
β-lipoproteins, ”	69	7.0	64	8.0	.5	57	6.4	1.2
Protein-bound hexose, ”	110	9.4	119	5.0	.8	103	44	.7
Hexosamine, ”	174	9.0	194	6.6	1.9	166	3.5	.8
Protein-bound CHO, ”	296	12.3	312	8.6	1.1	269	6.3	1.2
Albumin-bound CHO, ”	18	4.0	16	3.5	.4	13	3.5	.9
α-globulin-bound CHO, ”	205	7.5	222	7.7	1.6	191	5.3	1.5
β-globulin-bound CHO, ”	43	3.2	42	3.0	.2	35	2.0	2.2
γ-globulin-bound CHO, ”	22	1.6	33	2.6	3.6	27	2.0	2.0
CHO, % in α-globulin	12.3	.6	11.3	1.0	.8	11.3	.8	1.0
” , ” ” β-globulin	4.6	.4	5.2	.3	1.2	4.4	.5	.3
” , ” ” γ-globulin	4.8	.6	2.3	.2	4.0	4.7	.3	.2
α-globulin/γ-globulin	3.5	.3	1.2	.1	20.0	2.5	.3	2.3
$\frac{\text{CHO, \% in } \alpha\text{-globulin}}{\text{CHO, \% in } \gamma\text{-globulin}}$	3.4	.4	6.3	.9	9.4	2.9	.5	2.2

SEm is stand. error of the stand. dev.

$\frac{OD}{SE\text{-diff.}}$ is the observed difference between control and exp. group divided by stand. error of that difference (above 2.5 significant).

tively small increase prevents a more definite conclusion. Similarly the constancy of the beta globulin level and the small amounts of carbohydrate-bound albumin do not favor any calculation.

However, the contrast between the marked rise of serum gamma globulin with only a small elevation of its carbohydrate moiety, best reflected in the difference between the alpha globulin/gamma globulin ratio of protein and the ratio of the carbohydrate concentration of these proteins, permits the conclusion that the excess gamma globulin formed in tuberculosis is poor in carbohydrates. If calculations of the carbohydrate concentration of the excess gamma globulin in the tuberculous animals are permissible from the averages, this increment in the amount of 1 g per 100 ml has a carbohydrate moiety of almost 1% in contrast to the gamma globulin in control guinea pigs with a mean carbohydrate concentration of 4.8%. This is in contrast, for instance, to the elevated carbohydrate concentration of the excess gamma globulin in myeloma(17,18) and the unchanged concentration in infectious mononucleosis(19), experimental brucellosis(6) and hepatitis(20), and the small differences recently demonstrated by Müller-Eberhard and Kunkel(21) in the carbohydrate concentration in various components of gamma globulin in normal and abnormal circumstances. The findings in experimental tuberculosis suggest that the excess gamma globulin represents a reaction globulin(22) different in nature from the normal gamma globulin and possibly formed in other sites.

Summary. The previously demonstrated reduction of serum albumin and elevation of gamma globulin and mucoprotein in experimental guinea pig tuberculosis is associated with a slight elevation of total serum hexose

which is to the greatest extent accounted for by a slight rise of the carbohydrate containing alpha globulin. No evidence was obtained that the carbohydrate content of this small increment differs from that of the normal alpha globulin. In contrast the marked rise of gamma globulin is associated with an only very small increase of gamma globulin associated carbohydrates. This suggests that the excess gamma globulin in experimental tuberculosis differs from the normal gamma globulin by a very low carbohydrate content and suggests a different nature and possibly a different origin of this increment. The serum protein-bound lipids are not altered in experimental tuberculosis.

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1. Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.*, 1947, v26, 90.

2. Volk, B. W., Saifer, A., Johnson, L. E., and Oreskes, I., *Am. Rev. Tuberc.*, 1953, v67, 299.

3. Winzler, R. J., *Methods of Biochemical Analysis*, v2, Glick, D., Editor, Interscience Publishers, N. Y., p279-311.

4. Weimer, H. E., and Moshin, J. R., *Am. Rev. Tuberc.*, 1952, v68, 594.

5. Turner, G. C., Schaffner, F., Eshbaugh, D. E., and Huerga, J., *Dis. Chest.*, 1953, v23, 154.

6. Weimer, H. E., Boak, R. A., Carpenter, C. M., Moshin, J. R., Drusch, M. E., and Miller, J. M., *J. Inf. Dis.*, 1955, v96, 19.

7. Greenspan, E. M., *A. M. A. Arch.*, 1954, v93, 863.

8. Durrum, E. L., Paul, N. K., and Smith, E. R. B., *Science*, 1952, v116, 428.

9. Koiw, E., and Gronwall, A., *Scand. J. Clin. and Lab. Invest.*, 1952, v4, 244.

10. Sher, B. C., Czaja, Z. G., Takimura, Y., and Popper, H., *Am. Rev. Tuberc.*, in press.

11. Huerga, J. de la, and Popper, H., *J. Lab. and Clin. Med.*, 1950, v35, 459.

12. Huerga, J. de la, Dubin, A., Kushner, D., Dyniewicz, H., and Popper, H., *ibid.*, 1956, v47, 403.

13. Elson, L. E., and Morgan, W. T. L., *Biochem. J.*, 1933, v27, 1824.

14. Lustig, B., and Langer, A., *Biochem. Z.*, 1931, v242, 320.

15. Huerga, J. de la, Yesinick, C., and Popper, H., *Am. J. Clin. Path.*, 1953, v23, 1163.

16. Block, R. T., Durrum, E. L., and Zweig, G., *A Manual of Paper Chromatography and Paper Electrophoresis*, Acad. Press Inc., New York, 1955, p407.

17. Magalini, S. I., Stefanini, M., Marin, H. M., *Am. J. Med. Sci.*, 1956, v231, 155.

18. Sachs, D. S., Cady, P., and Ross, G., *Am. J. Med.*, 1954, v17, 662.

19. Magalhes, M. N. de, Magalini, S. I., Moschides, E., and Stefanini, M., *Clin. Res. Proc.*, 1956, v4, 89.

20. Schaffner, F., Scherbel, A. L., Lytle, R. I., *J. Lab. Clin. Med.*, 1956, v48, 551.

21. Müller-Eberhard, J. H., and Kunkel, H. G., *J. Exp. Med.*, 1956, v104, 253.

22. Marrack, J. M., and Moch, M., *J. Clin. Path.*, 1949, v2, 161.

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Increased Metabolism in Fat-Deficiency: Relation to Dietary Fat.* (22827)

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Elevation of metabolic rate is consistently observed in rats fed a diet devoid of fat. As early as 1931, Wesson and Burr made reference to increased basal and carbohydrate assimilatory rates(1), findings which were confirmed and extended by Burr and Beber(2). It has been pointed out that disturbance in energy balance as indicated by increase in basal oxygen consumption is probably the earliest and most fundamental manifestation of the fat-deficiency syndrome, being recorded within 7-14 days after the start of the experimental diet in rats(3,4).

The manifestations of fat-deficiency are corrected by the administration of linoleic acid in amounts too small to exert an influence as a metabolite (*i.e.* "fat as fat *per se*"), indicating the specific role of this fatty acid as an essential substance(5). It is the purpose of this presentation to report the effect on oxygen consumption and body weight of

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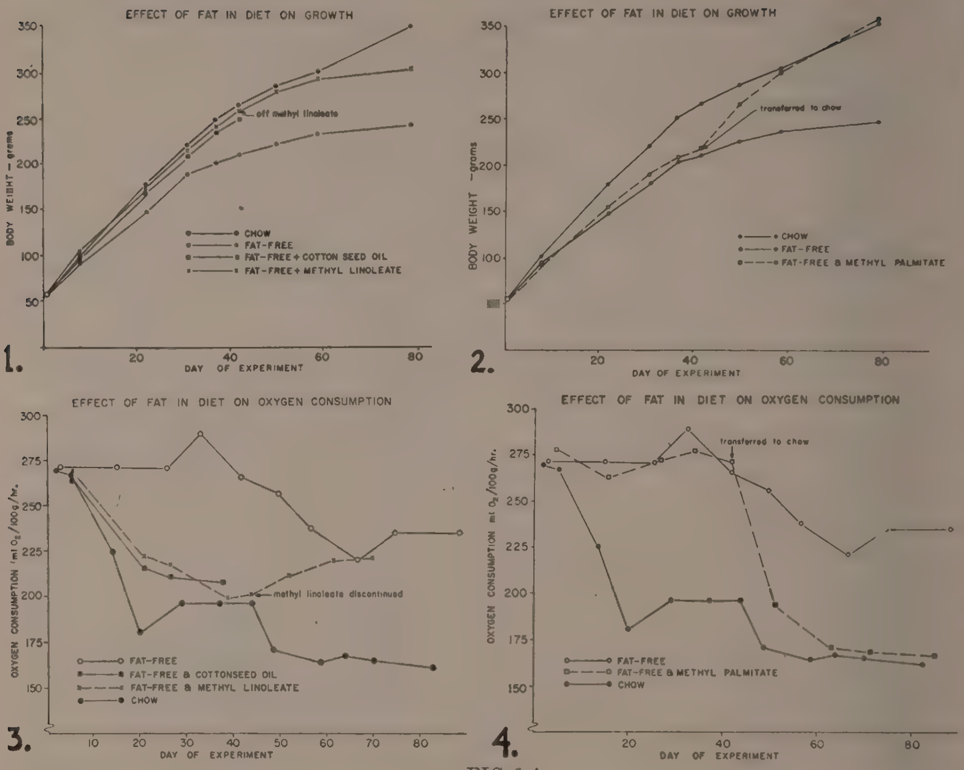

FIG. 1-4.

small amounts of saturated and unsaturated fatty acid preparations.

Materials and methods. Rats of the Holtzman strain were placed on experimental diets at weaning. Group I (12 rats) received the fat-free diet regularly used in this laboratory (3). Group II (12 animals) received the same fat-free diet supplemented by the daily administration for 6 weeks of 100 mg methyl linoleate[‡] directly into the mouth via syringe. Tocopherol was added to the methyl linoleate for antioxidant effect in ratio of 1 to 100. It became necessary to discontinue this supplement after 6 weeks because of unexpected unavailability. Group III (12 rats) was fed the same fat-free diet similarly supplemented by daily administration of 100 mg methyl palmitate. Original plans for transfer from the palmitate to methyl linoleate were impossible, and the animals were transferred to laboratory chow.[§] Group IV (12 rats) received the same fat-free diet similarly supplemented daily for 40 days with 200 mg cottonseed oil[||] which contains approximately 50% linoleic acid. Group V (15 rats) was fed laboratory chow. The animals were weighed weekly and observed for development of dermatitis. Oxygen consumption was determined weekly on 5 animals from each group according to a method previously described(3), modified by addition of a circulating pump. All determinations were carried out in an air-conditioned room with animals in a fasting, resting state; duplicate prolonged tracings were made after the animal had become motionless. The duration of the experiment was 11 weeks.

Results. The effects of various diets on growth are depicted in Fig. 1 and 2. It will be noted that supplementation with methyl linoleate resulted in gain entirely comparable to that achieved by animals fed laboratory chow, whereas an equal amount of saturated fatty acid (methyl palmitate) resulted in growth identical to that produced by feeding the fat-free diet alone. Following discontinuation of methyl linoleate, growth continued unaltered for approximately 20 days, after which it decelerated so that at 80 days, the mean weight of this group was significantly lower than that of chow-fed animals. In contrast, transfer of the methyl palmitate group to chow resulted in a pronounced acceleration in weight gain, which reached the level of chow-fed animals within a period of 20 days.

[‡] Obtained from Hormel Institute, Austin, Minn.

[§] Purina.

[||] Wesson.

The basal oxygen consumption rates in the group receiving linoleate and cottonseed oil supplements were not significantly different from that of chow-fed animals (Fig. 3). Following withdrawal of methyl linoleate, oxygen consumption returned gradually to the level of fat-free controls. Comparison of these findings with those shown in Fig. 4 reveals that supplementation of fat-free diet with methyl palmitate produced no change in oxygen consumption rate. Following transfer to laboratory chow, a pronounced decrease was noted after 10 days and within 20 days the rate was identical with that of the chow-fed animals.

Although characteristic dermatitis developed in fat-free animals after approximately 9 weeks, no such changes were observed in any other groups. In the group in which supplementation with methyl linoleate was withdrawn after 6 weeks, no skin changes were observed during the remainder of the experiment although significant alterations in oxygen consumption and weight gain were observed.

Discussion. The amount of methyl linoleate administered in these experiments constituted the equivalent of approximately 1.5% of the total daily caloric intake as fat. It seems clear that the effects on oxygen consumption and body weight cannot be attributed to the simple caloric contribution of linoleate but to the provision of a specifically required substance. This conclusion is supported by the failure of a calorically equal amount of methyl palmitate to influence either oxygen consumption or weight. Although absorbability of tripalmitin from the gastrointestinal tract is known to be poor(6), that of palmitic acid appears to be great enough(7) to substantiate the use of methyl palmitate as an experimental control.

Supplementation with cottonseed oil to provide 3% of the calories as fat resulted in changes essentially identical to those following methyl linoleate alone, indicating that the effects were due to the linoleic acid content. Deuel(8) has shown that the increase in weight of male rats receiving 50 mg methyl linoleate daily approached that found in animals receiving 53% of the calories as cottonseed oil and has suggested that larger doses, 100 mg or more, might maintain weight comparable to rats receiving the high fat diet.

Recently, Hansen and coworkers(9) have reported that infants fed to satiety with skim milk (1% calories as fat and 0.04% as linoleic acid) consumed more calories per kg of weight gain than did infants ingesting a standard milk mixture providing 38% of the calories as fat and 0.9% as linoleic acid. When the skim milk diet was supplemented daily with an amount of methyl linoleate equivalent to 1% of the calories, the daily caloric consumption per kg body weight decreased 20-30% without affecting weight gain or activity. These findings suggest that fat-deprivation increases metabolism in human infants as in rats, with resultant increased caloric requirements and intake. The implications of these observations on principles of infant feeding are obvious, particularly in view of the popularity of low-fat milk mixtures in clinical practice.

Summary. Disturbance in energy metabolism, as indicated by increased basal oxygen consumption and growth retardation, is prevented when rats receiving a fat-free diet are given daily supplements of methyl linoleate (100 mg) or cottonseed oil (200 mg). However, similar supplementation with a saturated fatty acid (methyl palmitate, 100 mg) in no way modifies the development of the fat-deficiency syndrome.

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1. Wesson, L. G., and Burr, G. O., *J. Biol. Chem.*, 1931, v91, 525.

2. Burr, G. O., and Beber, A. J., *J. Nutrition*, 1937, v14, 553.
3. Panos, T. C., and Finerty, J. C., *ibid.*, 1953, v49, 397.
4. ———, *ibid.*, 1954, v54, 315.
5. Burr, G. O., and Burr, M. M., *J. Biol. Chem.*, 1930, v86, 587.
6. Norcia, L. N., and Lundberg, W. O., *J. Nutrition*, 1954, v54, 491.
7. Swell, L., Trout, E. C., Field, H., Jr., and Treadwell, C. R., PROC. SOC. EXP. BIOL. AND MED., 1956, v92, 613.
8. Greenberg, S. M., Calbert, C. E., Savage, E. E., and Deuel, H. J., Jr., *J. Nutrition*, 1950, v41, 473.
9. Hansen, A. E., Wiese, H. F., Lawlis, M., Adam, D. J. D., Goldman, A., and Baughan, M., *Am. J. Dis. Child.*, 1955, v90, 621.

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Improved Procedures for Preparation of Tissues for Chemical Analyses.* (22828)

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Many years of work on histochemical characterization of various tissues have required different technics for their preparation for precise chemical analyses. Previously, some soft tissues were minced and aliquots of wet minced tissue were weighed for all analyses: skeletal muscle(1,2), brain(3) and heart(4); some tissues were minced, dried and smashed in a special apparatus for all analyses: liver (5,6), skin(7,8), kidney(9), cartilage(10,11).

The method described here has many advantages: it is saving in time and effort while producing material excellent for sampling; it permits a uniform routine for all soft and hard tissues, especially cartilage; and it provides for numbers of tissues being taken from the same animal simultaneously and handled more satisfactorily.

Procedure. Tissues are taken from anesthetized animals, wiped with dry gauze and dropped quickly into ground glass-stoppered jars. After being cooled in a refrigerator to minimize water loss, the tissues that have visible connective tissue or fat are placed on a tile and adherent structures removed. The tissues are then dropped into glass-stoppered weighing bottles and again cooled before mincing with points of scissors. Tissues such as liver and brain that do not have visible fat or connective tissue are simply cooled and minced. Skin after being trimmed is cut into strips 8 × 10 cm. Samples are allowed to come to room temperature, and then weighed into vycor crucibles in aliquots depending upon amount of wet tissue available. These samples can range to 10 g of wet tissue. It is preferable to weigh 2 or more samples of the same tissue to check determinations for water and fat. The crucibles are next placed in 100°C thermostatically controlled oven for 48 hours, after which they are cooled in a desiccator over activated alumina oxide and then weighed. After this, the dried tissues are covered with dry ethyl ether and placed in another desiccator containing sufficient ethyl ether to cover bottom of desiccator to about one inch. After remaining in this ether desiccator for 24 hours, the ether layer over tissue containing extracted fat, is removed with Pasteur pipet and new dry ethyl ether added. This process is repeated at least 3 times. After removal of last ether extraction, the crucibles are placed in 100°C oven for approximately 5 hours, then cooled in a desiccator over activated alumina oxide and weighed. This gives a dry, fat-free solid which can be kept in a desiccator until ready to be ground and analyzed.

Grinding the dry tissue. The mills used for grinding dry tissues were either a Zassen-

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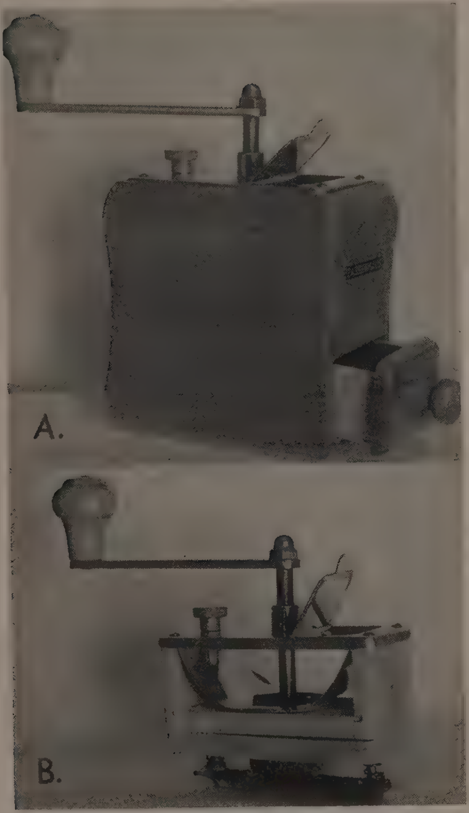

FIG. 1. A. Zassenhaus spice mill showing opened spring door through which tissue is introduced into mill and opened drawer that receives the ground sample. On top is also shown grinding handle and screw for setting size of desired particles. B. Cutting unit lifted out of mill.

haus spice or pepper mill,[†] depending on size or weight of tissue sample. These mills have hard steel cutters that can be adjusted for size of particles by a turn screw in top of spice mill or in bottom of pepper mill. The set screws on both ends of spice mill can be removed permanently so that the cutting unit can be lifted out readily. Illustrations of spice mill and cutting unit are shown in Fig. 1.

For grinding, dried tissue is scraped quantitatively with stainless steel spatula from the vycor crucibles onto a square of glazed paper and then transferred by brushing into the cutter compartment through a spring door on top of mill. The tissue is ground in a few minutes, requiring no effort. The powder is collected into a small drawer of the mill or in a devised cellophane bag. If the drawer is used, the powder from the drawer is transferred to a square of glazed paper, the cutters are lifted out and the drawer and the mill box are tapped sharply several times on the glazed paper until all particles of tissue have fallen from mill onto paper. The cutters are held over the glazed paper and any powder sticking to them is brushed with the sample for quantitative recovery. If the cellophane bag is used it is prepared as follows: a strip of heavy cellophane 18 × 6″ is wrapped around the cutter unit, using the extra for overlap; then bottom part of cellophane envelope is pleated and a hem is folded over to close the end of bag which is then fastened with a paper clip. For grinding, the cutting unit can either be returned to mill box for holding or it can be held firmly with a wood clamp attached to laboratory desk. When grinding is finished, cutter unit with attached cellophane bag is removed and placed on laboratory desk, the paper clip removed and the bag opened flat. Any adhering powder on cutters is brushed with the sample on the cellophane piece. The ground samples can be placed into small screwtop jars for later work, or they can be ground finer immediately.

To grind sample finer, samples from the mills are placed in a mullite mortar and either ground by hand or ground with a Fisher Mortar Grinder. The Fisher Electric Grinder is faster and superior to hand grind, requiring only about 15 minutes for soft tissues, and a little longer for cartilage samples. The powdered tissues are then returned to screw-top jars and, before weighing aliquots for analyses, they are dried in a 100°C oven overnight, cooled and kept in a desiccator over activated alumina oxide.

Summary. Improved procedures for preparation of tissues for chemical analyses have been presented. These procedures have definite advantages over all previous procedures; they are saving in both time and effort; they permit a uniform routine for all types of tissues; they produce homogenous materials that insure adequate sampling, and they enable numbers of tissues to be taken from the same experimental animal and handled satisfactorily.

[†] Kirkhams, Glens Falls, N. Y. Mill No. M495.

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1. Hastings, A. B., and Eichelberger, L., *J. Biol. Chem.*, 1937, v117, 73.

2. Eichelberger, L., Akeson, W. K., and Roma, M., *Am. J. Physiol.*, 1956, v185, 287.

3. Eichelberger, L., and Richter, R. B., *J. Biol. Chem.*, 1944, v154, 21.

4. Moulder, P. V., Eichelberger, L., and Roma, M., submitted for publication.

5. Eichelberger, L., *J. Biol. Chem.*, 1941, v138, 583.

6. Eichelberger, L., and McLean, F. C., *ibid.*, 1942, v142, 467.

7. Eichelberger, L., Eisele, C. W., and Wertzler, D., *ibid.*, 1943, v151, 177.

8. Eichelberger, L., and Roma, M., *J. Invest. Dermatol.*, 1949, v12, 125.

9. Eichelberger, L., and Bibler, W. B., *J. Biol. Chem.*, 1940, v132, 645.

10. Eichelberger, L., Brower, T. D., and Roma, M , *Am. J. Physiol.*, 1951, v166, 328.

11. Eichelberger, L., and Roma, M., *ibid.*, 1954, v178, 296.

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The Breeding System in *Stylonychia putrina*. (22829)

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Mating types have been reported in *Paramecium aurelia*(1-4), *P. bursaria*(5-7), *P. caudatum*(9-12), *P. multimicronucleatum*(8, 9), *P. trichium*(13), *P. calkinsi*(14), *Euplotes patella*(15), *Tetrahymena pyriformis*(16-18), *Stylonychia putrina*(19), and *Oxytricha bifaria*(20). In several of these species a number of sexually isolated varieties have been discovered. In some, the number of mating types found in each variety was 2, but in *P. bursaria, P. multimicronucleatum, P. trichium, E. patella, T. pyriformis, S. putrina,* and *O. bifaria* multiple mating types have been found. In one variety of *P. bursaria* there were eight; in *E. patella,* 6; in a variety of *T. pyriformis,* 11; in *S. putrina,* 5; and in *O. bifaria,* 9. The present paper reports another variety and additional mating types in the first variety of *S. putrina* reported in 1952 (19).

Materials and methods. Cultures of the 5 mating types in the first variety reported in 1952 died out in the laboratory. Two clones of progeny from crosses that had been made between clones of some of these 5 mating types were left. In 1952, 1954, and 1955, 155 additional clones of *Stylonychia putrina* were collected in Southern California in 6 different sites, 3 in the mountains and 3 in the valleys. The food was a liquid culture of a species of *Chlamydomonas* to which a loop of *Aerobacter cloacae* was added. The animals were isolated and grown in depression plates and later transferred to vials. The clones were fed every day, often twice a day.

Results. Mating behavior. The mating behavior found was that described previously (19), except that some degree of mating activity was observed in practically all of the 155 clones reported here, while none was observed in more than a third of the 41 clones reported in 1952.

Varieties and mating types. The two clones of progeny mentioned above reacted with some of the clones of a group that is called Variety I in this report. The mating types of Variety I now in existence have not been correlated with those previously reported. In 1954, six mating types were found in this variety. Various crosses were made among clones of these 6 mating types and among the progeny raised were 5 clones belonging to 3 new mating types. Among clones collected in 1955, 6 additional mating types were found, making a total of at least 15 mating types in Variety I. Eleven mating types were found among the Variety II clones, giving a total of 26 mating types for the two varieties.

The situation in *E. patella*(15) differed from that in *Paramecium* in that selfing was common in *Euplotes* and it was shown that some of the pairs found in conjugating mixtures consisted of 2 animals from the same clone. Also, animal-free fluid from a culture of one mating type induced selfing among animals of other mating types. Because of these differences, Sonneborn(3) concluded that the conception of a mating type in *Euplotes* was different from that in *Paramecium*. Selfing was uncommon in the 155 clones reported in this paper, but no method was found to determine the clonal origins of the animals associated in pairs. It is, therefore, not possible to state which of the two concepts of mating type applies to *S. putrina*.

Summary. Two sexually isolated varieties were found among 155 clones of *Stylonychia putrina* collected in Southern California plus 5 clones produced in the laboratory. In Variety I, at least 15 mating types have been found; in Variety II, 11 mating types.

The writer wishes to thank Dr. T. T. Chen for his help and guidance in this work.

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1. Sonneborn, T. M., *Proc. Nat. Acad. Sci.*, 1937, v23, 378.

2. ———, *Anat. Rec.*, 1942, v84, 542.

3. ———, *Advances in Genetics*, 1947, v1, 263.

4. Beale, G. H., and Schneller, M., *J. Gen. Microbiol.*, 1954, v11, 57.

5. Jennings, H. S., *Genetics*, 1939, v24, 202.

6. Jennings, H. S., and Opitz, P., *ibid.*, 1944, v29, 576.

7. Chen, T. T., *Proc. Nat. Acad. Sci.*, 1946, v32, 173.

8. Giese, A. C., *Anat. Rec.*, 1941, v81, supplement, 131.

9. Giese, A. C., and Arkoosh, M., *Physiol. Zool.*, 1939, v12, 70.

10. Gilman, L. C., *Biol. Bull.*, 1949, v97, 239.

11. ———, *ibid.*, 1950, v99, 348.

12. Hiwatashi, K., *Sci. Rept. Tohoku Univ. 4th Ser.*, 1949, v18, 137.

13. Sonneborn, T. M., *Collecting Net*, 1939, v14, 77.

14. Wichterman, R., *Proc. Penna. Acad. Sci.*, 1951, v25, 51.

15. Kimball, R. F., *Am. Nat.*, 1939, v73, 451.

16. Elliott, A. M., and Gruchy, D. F., *Biol. Bull.*, 1952, v103, 301.

17. Elliott, A. M., and Hayes, R. E., *J. Protozool.*, 1955, v2, 75.

18. Gruchy, D. F., *ibid.*, 1955, v2, 178.

19. Downs, L. E., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 605.

20. Siegel, R. W., *Biol. Bull.*, 1956, v110, 352.

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Influence of Steroids on Carbohydrate Content of Rabbit Kidney: A Chromatographic Study.[*] (22830)

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Intraglomerular kidney lesions produced in rabbits by the short-term administration of cortisone(1), prednisone and prednisolone are stained by the periodic acid-Schiff (PAS) method, a reaction of carbohydrates possessing free 1,2-glycol and amino alcohol groups (2). In the course of a systematic investigation into the nature of these renal lesions, chromatographic studies were designed to demonstrate the effects of cortisone acetate,[†] prednisone,[‡] and desoxycorticosterone acetate on concentrations of certain carbohydrates extractable from rabbit kidneys. The sugars studied, rhamnose, mannose, fucose, glucose and galactose, are known to stain with PAS (2).

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[†] Cortisone acetate was supplied through the courtesy of Mr. H. K. McLaughlin of Upjohn Co. of Canada.

[‡] Prednisone was supplied through the courtesy of Mr. J. W. Brisick, Schering Corp. Ltd. of Canada.

Materials and methods. New Zealand white male rabbits ranging from 2 to 2.8 kg were maintained for a control period of 7 to 14 days on Sherwood rabbit pellets *ad libitum*. The left kidney was removed under nembutal and ether anesthesia. Postoperatively, the animals were provided with the same diet, but drinking water was replaced by 1% sodium chloride solution. This regimen was continued for 3 weeks during which the animals were divided into the following treatment groups: Group A: Control; Group B: 5 mg of desoxycorticosterone acetate intramuscularly/day; Group C: 5 mg of desoxycorticosterone acetate and 7.5 mg of cortisone acetate intramuscularly/day; Group D: 5 mg of prednisone suspension intraperitoneally/day. After 21 days, the animals were killed by overdose of nembutal and were autopsied. Portions of right kidney were fixed in Zenker-Formol, sectioned at 2.5 μ and stained with PAS stain. The remaining kidney tissue was extracted with 0.5 N sodium hydroxide for 4 days at 4°C; after neutralization, the extract was treated with 2 volumes of cold ethanol to precipitate acid mucopolysaccharides (fraction 1). A second precipitate (fraction 2) was obtained by increasing the ethanol concentration to 84%. Deproteinization of these extracts was not attempted. The 2 fractions were hydrolysed with a cation exchange resin (Permutit Q), and hydrolysates subjected to paper chromatography as described by Glegg and Eidinger (2). In this technic, hexosamines and hexuronic acid were either destroyed or adsorbed by the resin and did not appear on the chromatograms. After development of the chromatograms and location of sugar areas with aniline hydrogen oxalate, the quantities of the various sugars present were estimated by comparing their spot intensities under ultraviolet light (3660 Angstrom units) with those resulting from subjecting 125 μg of standard sugars to the chromatographic procedure. In this manner it was possible to make a crude estimation of the quantities of sugar in milligrams per hundred grams of fresh kidney.

Results. Several areas in all kidneys were observed to stain positively with the PAS technic: (a) capillary basement membrane of the glomeruli (b) the brush border of cells lining the proximal convoluted tubules and (c) the cytoplasm of cells lining the convoluted and collecting tubules. In the control group and in animals treated with desoxycorticosterone acetate alone, no further staining areas were identified. In the groups which received cortisone and prednisone, characteristic nodular glomerular lesions, some of which stained with PAS, were observed. A moderate increase in the quantity of PAS-staining material was noted in the capillary walls and in intratubular casts. These lesions were identical to those described by others(1,3). The pathological alterations were more numerous and severe in the group treated with prednisone.

Fig. 1 indicates the concentrations of rhamnose, mannose, fucose, glucose and galactose in fractions 1 and 2 in each of the groups. The most notable alterations were the disappearance of rhamnose from the kidneys of the groups treated with cortisone acetate or prednisone and a diminution in concentration in those treated with DCA. There was an increase in mannose and galactose content in all groups treated with steroids, but the levels of fucose and glucose were not significantly changed.

Discussion. Sommers and Haley(4) noted the accumulation of an abnormal substance in the glomerular stroma, capillaries, and walls of kidney arterioles in humans and ham-

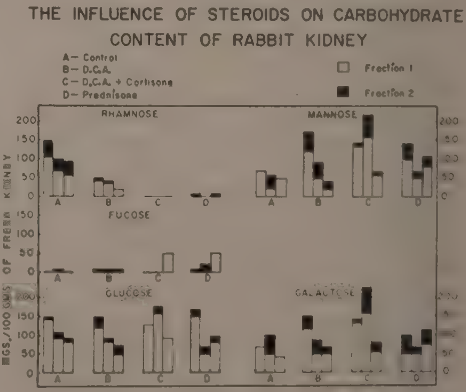

FIG. 1.

sters treated with cortisone. These changes are similar qualitatively to those which have been reported in human diabetic glomerulosclerosis(5). The abnormal substance was felt to be a mucopolysaccharide, since it is removed by incubation with hyaluronidase.

These findings coupled with those described above indicate an influence of adrenal steroids on the carbohydrate content of the kidney which may represent a link in the pathogenesis of glomerulosclerosis in human diabetes.

Summary. The changes induced in concentration of rhamnose, mannose, fucose, glucose and galactose in the kidney of rabbits treated with various steroids were studied using a semi-quantitative chromatographic method. The findings are discussed in relation to the pathogenesis of diabetic glomerulosclerosis.

1. Wilens, L. S., Stumpf, H. H., *Am. J. Path.*, 1952, v31, 275.

2. Glegg, R. E., Eidinger, D., LeBlond, C. P., *Science*, 1954, v120, 839.

3. Bloodworth, J. M. B., Hamwi, G. J., *Am. J. Path.*, 1955, v31, 167.

4. Sommers, S. C., Haley, K. H., PROC. SOC. EXP. BIOL. AND MED., 1956, v91, 919.

5. Sommers, S. C., Crozier, R., Warren, S., *Am. J. Path.*, 1954, v30, 919.

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A New Colorimetric Reagent for Micro Determination of Ammonia.* (22831)

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This paper describes a new colorimetric procedure for the measurement of ammonia in quantities of 0.05 to 0.5 micromole. The method has proved to be more rapid and convenient than the usual microtitration, and the precision appears to be at least as good. The microdiffusion method of Conway(1) is employed to separate the ammonia from interfering substances. The subsequent measurement is based on the well-known reaction of ammonia with hypobromite. A carefully measured volume of hypobromite solution is added, enough so that an excess is present. The hypobromite remaining after the oxidation of the ammonia is then measured by its power to decolorize a dilute solution of phenosafranin. Previously described methods based on the reaction of ammonia with hypobromite depend upon iodometric titration of the excess hypobromite. In one such procedure, that of Levy and Palmer(2), as little as 0.36 micromole of ammonia is oxidized by hypobromite in a volume of 2 ml. For studies of ammonia in blood and tissues, however, it is desirable to work at still greater dilutions. As a first step, therefore, the iodometric method was used to test the reaction at greater dilutions. It was thus established that the oxidation of ammonia by hypobromite is stoichiometric at ammonia concentrations at least as low as .03 micromole per ml.

The choice of phenosafranin as a colorimetric reagent was based on tests of a number of dyes and stains. Although hypobromite has a decolorizing action on many such intensely colored substances, it was found that in most instances the decolorizing action is not directly proportional to the hypobromite concentration. With phenosafranin, however, a strictly linear relationship obtains.

Methods. *Borate buffer,* 0.5 *M*, pH about 9.8. A 16 g quantity of sodium hydroxide is dissolved in 800 ml water, 31 g boric acid is added and dissolved, and the solution is diluted to 1 l. *Phenosafranin,* approximately 0.006%. It is convenient to prepare a liter of 0.02% solution and to make dilutions as required. The concentration should be so ad-

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justed that when 1 ml is added to 7.5 ml water and 1 ml of borate buffer, the optical density at 515 mμ is about 0.9. The phenosafranin solution is very stable. *Bromine solution.* While this can be prepared from liquid bromine and potassium bromide, a more convenient method is as follows: 16 g potassium bromide and 1.67 g potassium bromate are dissolved in 450 ml water. An 8.5 ml volume of 10 N sulfuric acid is added and the solution is diluted to 500 ml. It should be stored in a dark place. The bromine concentration slowly decreases. *Hypobromite reagent.* To 25 ml of the borate buffer is added about 0.5 ml of the bromine solution. The concentration should be so adjusted that when 1 ml of phenosafranin is added to a mixture of 2.5 ml water and 1 ml of hypobromite, with subsequent dilution to 9.5 ml, the optical density at 515 mμ is between 0.1 and 0.2. The hypobromite should be freshly prepared on the day of use. A slow change to the inactive bromate occurs, amounting to about 1% per hour.

Procedure. Microdiffusion is carried out as described by Conway(1), ammonia being absorbed in 1.5 ml of 0.01 N sulfuric acid in the central chamber. At end of diffusion period the contents of this chamber are transferred to colorimeter tube by a dropper. The chamber is washed with 1 ml water which is transferred to the colorimeter tube as completely as possible. To the solution in the tube (volume 2.5 ml) is added 1 ml of hypobromite solution. After at least 2 minutes, 1 ml of phenosafranin is added. After at least one minute, 5 ml of water is added. The solution is very thoroughly mixed after each addition. The optical density is read at 515 mμ. Results are calculated from a calibration curve prepared from standard ammonium sulfate solutions subjected to microdiffusion procedure. All readings are taken with instrument set so that distilled water gives an optical density of zero (100% transmittance). Blank determinations are run in triplicate with each group of samples, and for each ammonia determination the average blank value is subtracted from the reading. Care must be taken to insure that amount of ammonia in the aliquot of sample is less than the amount of hypobromite added, since the curve becomes a horizontal line when the hypobromite is completely reduced by ammonia (Fig. 1). If the reading approaches the upper limit, the determination should be repeated, if possible, on a smaller aliquot.

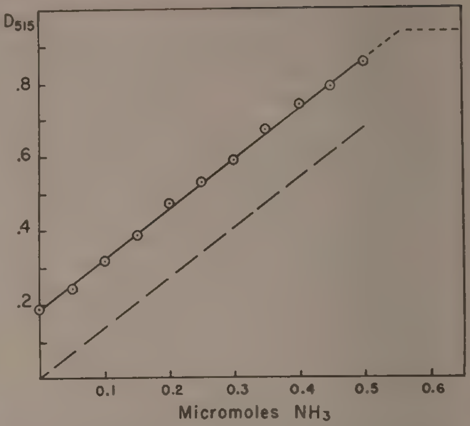

FIG. 1. Standardization curve. Horizontal portion at upper end of curve (dotted line) indicates color intensity of phenosafranin when no hypobromite is added, or when all of the hypobromite is reduced by ammonia. Dashed line represents curve after subtraction of the blank from each reading.

Results. Fig. 1 shows a standardization curve done on ammonium sulfate solutions directly, without microdiffusion. Readings were taken with a Coleman 6 B instrument. The dashed line represents the curve obtained after subtraction of the blank value from each reading. The points on the curve represent individual determinations done in one group, and the small deviations from the curve suggest the degree of precision which can be attained. The magnitude of the blank varies from one run to another, being dependent upon the concentration of hypobromite, but the slope of the curve is highly reproducible.

Application to brain tissue. The tissue (obtained after freezing the brain *in situ*) is ground and extracted with cold 10% trichloroacetic acid, using approximately 5 ml/g. After filtration, 1 ml aliquots of the extract are used for the determination. The microdiffusion must be started immediately, and is carried on for a 90-minute period. Correction for the slow formation of ammonia from glutamine during microdiffusion is made as described by Richter and Dawson(3).

Application to blood. For measuring blood ammonia it is desirable to reduce the range of the method. The concentrations of hypobromite and of phenosafranin are reduced by about one-third. The volumes of reagents used are not changed, but the final dilution is made with only 1.5 ml water, giving a final volume of 6 ml. Blood is treated with ammonia-free potassium oxalate to prevent clotting, and the determinations are carried out on 1 ml aliquots. These may be pipetted into the Conway units and the diffusion started within 5 minutes after the blood is drawn, or more elaborate precautions may be taken to avoid ammonia formation after shedding(1). The diffusion period is limited to 20 or 30 minutes, in order to minimize ammonia formation due to action of the alkali on other blood constituents. The calculations are based on standards treated in the same way, since recovery is much below 100% in these short diffusion periods. Conway's(1) correction factors may be applied to allow for ammonia formation after shedding and for that due to action of the alkali, and for the slightly lower rate of diffusion from blood-carbonate mixtures as compared with water-carbonate standards.

Glutamine. The colorimetric method has been applied to the measurement of glutamine in brain tissue. One ml of the trichloroacetic acid extract is diluted with 4 ml of 10% trichloroacetic acid, stoppered loosely, and heated at 70° for 75 minutes. The glutamine is thereby converted to ammonia and pyrrolidonecarboxylic acid (method of Richter and Dawson(3)). The total ammonia is determined on 1 ml aliquots, and the value for preformed ammonia is subtracted.

Non-protein nitrogen. The method has been applied to the direct determination of non-protein nitrogen without microdiffusion. A suitable aliquot of a trichloroacetic acid filtrate of blood or tissue is digested by the Koch-McMeekin method with the precautions recommended by Miller and Miller(4). After the last addition of hydrogen peroxide, the heating is continued for 40 minutes to insure complete removal of hydrogen peroxide. (This is a necessary precaution, since any hydrogen peroxide remaining would react with the hypobromite). After cooling, the digest is diluted and enough sodium hydroxide is added to almost, but not quite, neutralize the sulfuric acid present. (The amount of sodium hydroxide necessary may be determined on a blank digest.) The solution is then made up to a definite volume and aliquots are taken for the determination. Blanks are run on the digestion procedure, and the calculations are based on standards not subjected to microdiffusion.

Summary. A new colorimetric procedure is substituted for microtitration in the measurement of ammonia by the Conway method. The ammonia is oxidized by addition of hypobromite, and the excess hypobromite determined by its power to decolorize phenosafranin. The range of the method as described is 0.05 to 0.5 micromole. The procedure has been applied to brain tissue and to blood, and to the measurement of glutamine. It has also been used for the determination of non-protein nitrogen after Koch-McMeekin digestion, the microdiffusion being omitted.

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1. Conway, E. J., *Microdiffusion Analysis and Volumetric Error,* 3rd ed., London, Crosby Lockwood and Sons, 1950.

2. Levy, M., and Palmer, A. H., *J. Biol. Chem.,* 1940, v136, 57.

3. Richter, D., and Dawson, R. M. C., *ibid.,* 1948, v176, 1199.

4. Miller, G. L., and Miller, E. E., *Anal. Chem.,* 1948, v20, 481.

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Ventricular Arrhythmias and Automaticity following Norepinephrine.* (22832)

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In human subjects norepinephrine was shown to increase peripheral vascular resistance without accelerating the heart rate(1). Norepinephrine has also been reported to be less effective in precipitating ventricular ectopic rhythms(2) and it is being used in treatment of secondary shock when epinephrine is contra-indicated to restore arterial blood pressure. Recently, norepinephrine was observed to increase the vulnerability of the ventricles to fibrillation and the greater safety reported for norepinephrine was considered to be due to its slower rate of infusion in clinical practice(3). Because norepinephrine is employed extensively in treatment of hypotensive states, this study was undertaken to compare its potency relative to that of epinephrine in eliciting experimental, ventricular rhythms. Using cats under pentobarbital, harman methosulfate was given to predispose the heart to ventricular rhythms by a method previously described(4). To extend the quantitative comparison of norepinephrine and epinephrine on cardiac excitability, their actions on isolated cardiac papillary muscle preparation of Cattell and Gold(5) were also investigated.

Methods. Experimental ventricular arythmias. The electrocardiographic changes produced by epinephrine and norepinephrine were studied in normotensive cats under sodium pentobarbital anesthesia. Harman methosulfate (2.5 mg/kg) was injected 5 minutes before epinephrine or norepinephrine and the ensuing electrocardiographic changes were recorded. *Automaticity studies.* Following the technic of Cattell and Gold(5) isolated, cat, cardiac papillary muscles were mounted in lucite holder and immersed in 75 ml of modified Locke's solution(6). The chordae tendinae were connected by a fine silk thread to a thin steel plate on each side of which a Baldwin SR-4 strain gauge was mounted and contractions were recorded with the strain gauge amplifier of a Sanborn Twin-Viso Cardiette. The muscle was stimulated electrically with a square wave shock from a thyratron stimulator at 60 per minute and the amplitude of contractions and threshold of excitability were noted. The incidence of automaticity (rhythmic contractions without electrical stimulation) was observed following addition of norepinephrine and epinephrine with equilibration period of at least 15 minutes between drugs.

Results. Nodal rhythm (NR), ventricular extrasystoles, tachycardia and flutter (VR) and ventricular fibrillation (VF) occurred following injection of both epinephrine and norepinephrine in cats previously treated with harman methosulfate (Table I). Immediately before the onset of fibrillatory rhythm, a migration of the T-wave into the QRS could be seen (Fig. 1). A similar interruption of T-waves by R-waves was observed by Fastier to occur before "epinephrine syncope" following benzene or furfurin(7). In contrast to larger mammals, cats were able to survive relatively long periods of ventricular fibrillation. Based on both the duration of arrhythmias and the incidence of ventricular fibrillation, norepinephrine was found to be significantly more active than epinephrine in eliciting ventricular ectopic rhythms ($p < 0.05$). *Automaticity of papillary muscles.* A pos-

TABLE I. Incidence and Duration of Arrhythmias in Cats Treated with 2.5 mg/kg Harman Methosulfate.

Drug	Dose, µg/kg	Number Total	NR	VR	VF	Duration of arrhythmias, sec.*
Epinephrine	2.5	10	0	6	3	84 ±3
	5.0	10	2	9	6	54 ±16
	10.0	11	3	11	5	78 ±20
	20.0	10	4	10	7	66 ±9
Norepinephrine	1.2	10	1	10	4	82 ±43
	2.5	10	0	9	4	116 ±40
	5.0	10	3	10	6	198 ±30
	10.0	10	4	10	9	162 ±16

* Mean ± S.E.

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* This study was supported in part by grant from National Research Council of Canada.

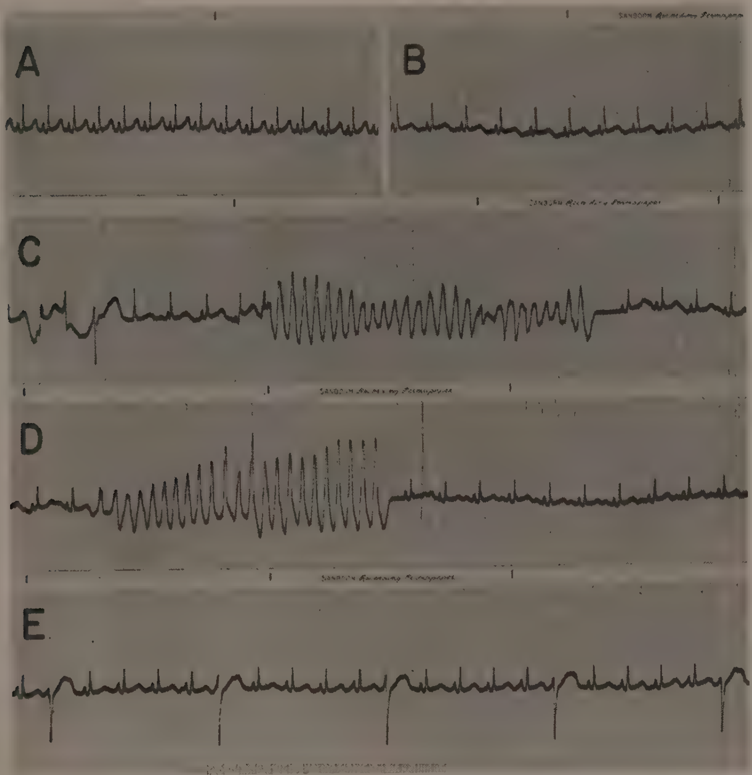

FIG. 1. Electrocardiograms of cat (2.4 kg) under sodium pentobarbital. A, 11:37 a.m., control reading. B, 11:44 a.m., 4 min. after harman methosulfate 2.5 mg/kg intrav. C, 11:45 a.m., 12 sec. after norepinephrine bitartrate 5 μg/kg intrav. D, continuation of C. E, 11:47 a.m., 2 min. after norepinephrine bitartrate.

sible relationship between ability of a substance to initiate automaticity and the property of precipitating ventricular ectopic beats was previously suggested(4). A comparison of the incidence of automaticity in the papillary muscles after addition of epinephrine and norepinephrine was therefore indicated to see whether norepinephrine was also more active in this preparation. As shown in Fig. 2, norepinephrine was found to be effective in increasing contractile force and inducing rapid, automatic contractions of papillary muscle. Although changes of contractile force of the papillary muscle produced by norepinephrine and epinephrine did not differ, the incidence of automaticity after norepinephrine was significantly greater than after epinephrine ($p < 0.05$).

TABLE II. Effects of Epinephrine and Norepinephrine on Cardiac Papillary Muscles.

Drug	Conc.	Incidence of automaticity	Mean % increase in amplitude of contractions
Epinephrine	$.4 \times 10^{-6}$	2/12	60
	1.6 "	6/12	123
Norepinephrine	.2 "	5/12	66
	.8 "	8/9	95

To compare the cardioaccelerator properties of epinephrine and norepinephrine with their effects on cardiac excitability, the increases in heart rate in isolated, perfused cats' hearts were determined. The tachycardia following epinephrine was greater than for equivalent doses of norepinephrine although the difference in activity was not significant. Thus, the relative effects of these substances

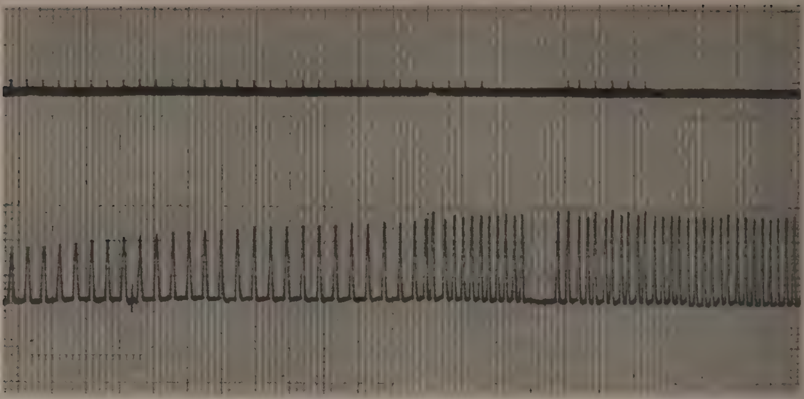

FIG. 2. Increase in contractile force and automaticity induced by norepinephrine bitartrate (1.6×10^{-6}) in the cat's heart papillary muscle. Upper record, stimulus marker (frequency, 1/sec.; duration, 10 millisec.; 15 volts). Lower record, contractions recorded by a strain gauge and amplifier of a Sanborn Twin-Viso Cardiette. Five horizontal lines represent 1 g.

on the sinus node and on ventricular excitability appeared to be separable.

Discussion. Electrocardiographic changes consisting of heart block and supraventricular rhythms were occasionally observed in normal subjects after intravenous norepinephrine(9). In cases of shock, on the other hand, most authors have found no evidence that the drug increased myocardial irritability during the hypotensive phase even after coronary occlusion. Nevertheless, the high potency of norepinephrine in precipitating ventricular rhythms in normotensive cats treated with harman methosulfate as well as in increasing cardiac irritability(8) points to a potential hazard of this pressor amine in clinical practice. Both epinephrine and norepinephrine were found to be less likely to elicit ventricular rhythms including fibrillation in dogs under cyclopropane which were made hypotensive by bleeding(2). The failure of norepinephrine to provoke arrhythmias in the therapy of shock in which the heart is vulnerable to fibrillation may therefore be related to the low blood pressure levels in these cases and not to the fact that norepinephrine lacks the effects of epinephrine on ventricular excitability.

Summary. (1) Nodal rhythm, ventricular extrasystoles, flutter and fibrillation were precipitated by norepinephrine in cats, under pentobarbital anesthesia, treated with harman methosulfate. Migration of the T-wave into the R-wave at onset of ventricular fibrillation was similar to that described before epinephrine induced arrhythmias. (2) The effects of norepinephrine on cardiac excitability were confirmed using isolated papillary muscles in which norepinephrine had a greater potency than epinephrine in eliciting automatic contractile activity.

1. Goldenberg, M., Pines, K. L., Baldwin, E. deF., Greene, D. G., and Roh, C. E., *Am. J. Med.*, 1948, v5, 792.

2. Brockman, H. L., and Huggins, R. A., *Arch. int. pharmacodyn.*, 1954, v99, 395.

3. Hoffman, B. F., Siebens, A. A., Cranefield, P. F., and Brooks, C. McC., *Circulation Res.*, 1955, v3, 140.

4. Hutcheon, D. E., Scriabine, A., and Rapuzzi, A. L., *J. Pharmacol.*, 1955, v113, 29.

5. Cattell, McK., and Gold, H., *ibid.*, 1938, v62, 116.

6. Chenoweth, M. B., and Koelle, E. S., *J. Lab. and Clin. Med.*, 1946, v31, 600.

7. Fastier, F. N., *J. Physiol.*, 1951, v112, 359.

8. Greiner, T. H., and Garb, S., *J. Pharmacol.*, 1950, v98, 215.

9. Moyer, J. H., Skelton, J. M., and Mills, L. C., *Am. J. Med.*, 1953, v15, 330.

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Serum Transaminase Activity in X-Irradiated Rabbits. (22833)

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Increased levels of serum glutamic oxaloacetic transaminase (G-O-T) activity after crushing and tourniquet injury to the rabbit leg were observed in our laboratories and will be reported elsewhere. Preliminary studies indicated that x-irradiation of left chest wall of an otherwise completely shielded rabbit in dosages of 2,500 r and higher, resulted in serum G-O-T elevations as early as 3 hours' post-exposure. It became of immediate interest, therefore, to determine if this very early response could be identified in rabbits after low dosage, whole body x-irradiation.

Methods. Rabbits of New Zealand strain, as in previous studies of traumatic injury(1), were employed. The animal, tied to a board, was placed 135 cm from center line of tube head. Dose levels of 500, 750, and 1,000 r were studied; half the dose was given ventrally and half dorsally in each instance.

Results. At the 500 r level, total irradiation time was 16.1 minutes. In another experiment, an attempt was made to determine if any relationship existed between initially elevated serum G-O-T activity and survival time of the animal.

The combined effects of bleeding and restraint on serum G-O-T activity were recorded in sham controls tied to the board in radiation position, without exposure, and bled serially along with x-rayed rabbits. Bleeding was accomplished by incising the ear vein, and transaminase determinations(2) were made on unhemolyzed sera after separation by low-speed centrifugation. The results are listed in Table I.

It is apparent that bleeding and restraint induce a transitory serum G-O-T activity increase that returns to normal values at 24 hours. However, the data demonstrate that a significant increase in serum G-O-T activity can be identified in the rabbit within 6 hours after 500 r of x-irradiation. At the 750 r and 1,000 r dose levels this alteration was recorded at 3 hours post exposure.

Perhaps the most striking aspect of transaminase change is its discriminating power on an individual basis. Of the 25 animals in the 750 r and 1,000 r experimental groups, 20 survived through 6 hours. In 15 of these 20, the 6-hour G-O-T activity ranged from 23 to 189 units above pre-irradiation levels. In 5 rabbits that died between 3 and 6 hours post-irradiation, the 3-hour determinations ranged from 22 to 196 G-O-T activity units above pre-irradiation values. In 15 sham control rabbits, on the other hand, the maximum change from "pre-irradiation" values at 6-hour sampling was 26 units (one animal) —the next highest change was 17 units (also a single rabbit). At the 500 r level, the significantly increased mean value for the group at 6 hours post-irradiation is not associated with much individual discrimination. Here, only 3 out of 12 rabbits recorded 6-hour G-O-T activity increases in excess of 17 units, although another 5 animals fell in the 14-16 units increase range.

The data in Table II indicate that there exists little relationship between the serum G-O-T change after 625 r of x-irradiation and survival time. The increase of 59 units, within the first 24 hours, recorded for rabbit No. 6 was associated with death on 8th day post-exposure. However, rabbit No. 9 died on 13th day with an observed change of −5 G-O-T units, while animal No. 5 survived the full 30-day term after an observed 24 unit increase within the first 24 hours. It would seem that although the initially elevated serum G-O-T activities may have resulted from increased permeability of irradiated tissue cell membranes, and thus more or less quantitatively reflect the extent of tissue damage, survival of the animal depends, in the main, on its ability to overcome and repair the insult.

Summary. Rabbits were subjected to x-

TABLE I. Serum Transaminase—Pre-irradiation and at Intervals Post-exposure. Means and standard errors.

	Pre-irradiation		+ 3 hr		+ 6 hr		+ 24 hr	
	N	Units/ml	N	Units/ml	N	Units/ml	N	Units/ml
Sham controls	15	22 ± 1.3	15	35 ± 2.2	15	30 ± 2.2	15	24 ± 1.7
500 r	12	24 ± 0.9	12	33 ± 1.3	12	38 ± 2.7*	11	31 ± 2.7*
750 r	12	22 ± 2.7	12	65 ± 14.1*	9	50 ± 5.3*	6	45 ± 7.9*
1,000 r	13	26 ± 1.7	12	52 ± 5.6*	11	66 ± 13.5*	8	42 ± 3.7*

* Difference statistically significant. Difference between means of sham controls and experimentals exceeds the stand. error for the difference by 2 + times.

TABLE II. Serum Transaminase and Time of Death (625 r Total Body Exposure).

Rabbit No.	Pre-irrad., units/ml	Max value 6-24 hr post-irrad., units/ml	Δ, units/ml	No. days 'til death
1	36	40	+ 4	12
2	26	50	+24	S* 30 d
3	30	50	+20	S 30 d
4	25	73	+46	21
5	26	51	+25	S 30 d
6	28	87	+59	8
7	29	43	+14	7
8	26	39	+13	S 30 d
9	38	33	− 5	13
10	15	50	35	8

* S = Survived.

irradiation in dosages of 500, 750, and 1,000 r. Serum glutamic oxaloacetic transaminase estimations were accomplished at 3, 6, and 24 hours post-irradiation. A significant increase in G-O-T activity could be identified at 6 hours in the rabbit group subjected to 500 r, and as early as 3 hours post-irradiation in the 750 r and 1,000 r animal groups. For the latter animals, the noted increase at 6 hours served to segregate 75% of the group on an individual basis. In rabbits subjected to 625 r of x-irradiation no relation was found between the serum G-O-T elevation and survival time.

1. Milch, L. J., Stinson, J. V., and Albaum, H. G., *Radiation Res.*, 1956, v4, 321.

2. Karmen, A., Wroblewski, F., and LaDue, J. S., *J Clin. Invest.*, 1955, v34, 126.

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Pathogenesis of Experimental Arteriosclerosis in the Rat.* (22834)

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We have demonstrated that the standardized production of severe arteriosclerosis (Mönckeberg type) and myocardial necrosis by means of renal injury in the albino rat can be completely prevented by prior thyroparathyroidectomy(1). Studies directed towards elucidation of the mechanism underlying this striking protective effect of thyroparathyroidectomy, led to the conclusion that the cardiovascular injury of intact rats was caused primarily by autointoxication with parathyroid hormone. On further analysis it was inferred that excess function of the parathyroid glands following standard renal damage was mediated through the adrenal cortex. This communication describes experiments which formed the basis for these conclusions, and outlines our present concept of the mechanism of experimental arterial mediosclerosis.

Procedures. The method used for production of lesions has been described previously (2). The procedure consists in a single par-

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enteral injection of the sodium salt of a poorly soluble sulfonamide in excessive dosage. This induces invariably massive and long-lasting intrarenal deposition of sparingly soluble crystals and results in obstructive nephropathy and permanent kidney damage, followed by severe disseminated cardiovascular and smooth muscle lesions in 4 to 7 days. The pathologic-anatomic changes are readily recognizable with the naked eye. Rats with typical injury manifest enlargement of parathyroids and adrenals as well as atrophy of thymus gland. Animals permited to survive for longer periods (2 to 5 weeks) show apparently no substantial progression of the injury but correspondingly increasing degrees of reparative changes in damaged organs such as scar formation and intense calcification of necrotic areas. Since emergence of these severe cardiovascular lesions could be obviated by prior thyro-parathyroidectomy, and since, in preliminary observations, a similar protective effect was achieved by adrenalectomy(3), further analysis of the mechanism responsible for disseminated muscular necrosis concerned the relative importance of the separate thyroid and parathyroid hormones and their possible relationship to the adrenal cortex. Three hundred adult male and female albino rats were used. In addition to thyro-parathyroidectomy, hypophysectomy and nephrectomy, the following procedures were carried out: Surgical removal of parathyroids and adrenals separately as well as in combination, elective and graded hormone substitution in such animals and in thyro-parathyroidectomized rats, and finally chemical "thyroidectomy," using 0.01% propylthiouracil in drinking water for a minimum of 4 weeks preceding the experiment. For hormonal substitution or treatment, cortisone and desoxycorticosterone acetate were injected subcutaneously in daily dosages of 3 mg each, starting 3 days prior to initiation of renal injury and continuing for a minimum of 3 days thereafter. Similarly, parathyroid extract was injected subcutaneously for substitution or poisoning in daily dosages ranging from 10 to 200 International Units, starting 5 days prior to kidney block and continuing for at least 5 days thereafter. Serial microdeterminations of calcium(4) and inorganic phosphate(5) were done in plasma of rats from most experimental groups. Complete postmortem examinations were performed on every animal. All important organs were fixed in formalin and sectioned for microscopic study using routine and special stains.

Results. Distribution of 9 sub-groups comprising 215 rats with various hormonal deficiencies and type of hormone employed are shown in Table I (Groups 2-11). For conciseness, experimental results are presented only for medionecrosis and calcification of the arterial tree as the severest and most consistent lesion (93% in intact rats, Group 1). However, data on vascular damage are fully in line with those for muscular necrosis in heart and gut.

Table I shows that treatment of thyroparathyroidectomized rats with parathyroid extract in amounts of 50 units daily induced typical vascular damage (Group 3). Since even the smallest dosage (10 units) raised plasma calcium levels to normal and allowed for development of nephrocalcinosis, parathyroid substitution was believed to be more than adequate so that these rats could be considered as thyroidectomized only. It is clear, therefore, that vascular necrosis did occur in complete absence of the thyroid gland. Similarly, chemical "thyroidectomy" did not prevent vascular injury, although it did provide a substantial reduction of its incidence (Group 4). The lowered incidence was interpreted as due to diminution of parathyroid hormone formation in the absence of thyroid hormone.

The singular significance of parathyroid hormone in pathogenesis of arterial necrosis is illustrated by the full protection afforded the 30 rats of Group 5 by parathyroidectomy alone. Moreover, one can readily enlarge this number by adding the 29 rats of Group 2, and, with some reservations, the 14 animals of Group 9. Thus, none of a total of 73 parathyroprive rats showed vascular lesions or other muscular necrosis under careful microscopic examination. Exhibition of parathy-

TABLE I. Hormonal Influences upon Development of Renogenic Arterial Medionecrosis in Albino Rats. (215, 4- to 6-mo-old animals of both sexes, with standard renal injury.)

Group No.	Endocrine status prior to hormone admin.	Hormone treatment or substitution daily	No. in group ♂	♀	Total	No. with arterial necrosis	Incidence of arterial necrosis, %
1	Intact	—	31	15	46	43	93
2	TP X	—	24	5	29	0	0
3	TP X	PE (10-20 units)		5	5	0	
		PE (50 ")		5	5	2	
4	"T X"	—	4	8	12	3	25
5	P X	—	22	8	30	0	0
6	P X	PE (100 units)	18		18	13	72
7	A X	Cortisone	18	6	24	0	0
8	A X	Cortisone & DCA	10	10	20	12	60
9	A X & P X	*Idem*	7	7	14	0	0
10	A X	Cortisone & PE (200 units)	12		12	8	67

X = Removal of gland, T = Thyroid, P = Parathyroid, A = Adrenal.
PE = Parathyroid extract, DCA = Desoxycorticosterone acetate.

roprive rats to excess amounts of parathyroid extract (100 I.U. daily) restored their ability to develop the characteristic lesions, as depicted in Group 6. Of 18 rats in this group, 13 or 72% presented typical vascular damage.

It was postulated, therefore, that autointoxication with hormone from excessively secreting parathyroid glands was responsible for emergence of muscular necrosis in intact rats with renal injury, (Group 1). Further evidence was seen in behavior of plasma calcium and phosphate levels of these animals in the initial stages of renal disease, in which calcium levels were normal or even elevated (9-15 mg%) in the face of marked retention of inorganic phosphate (8-12 mg%).

In further pursuing the role of parathyroid gland, rats deprived of their adrenals and maintained on cortisone did not incur parathyroid hypertrophy, and, like parathyroidectomized rats, failed to develop the characteristic picture of disseminated muscular necrosis (Group 7). However, when adrenalectomized animals were given DCA in addition to cortisone (Group 8), the parathyroid glands became greatly enlarged and typical cardiovascular and smooth muscle necrosis appeared. (Almost identical observations were made with hypophysectomized rats, not shown in Table I.) On the other hand, when adrenalectomized animals were, in addition, deprived of their parathyroid glands, DCA administration appeared to have lost its deleterious effect (Group 9), whereas typical muscular injury could be readily produced in adrenalectomized rats given parathyroid extract (200 I.U. daily) *instead* of DCA (Group 10).

Under our experimental conditions, therefore, desoxycorticosterone administration in itself to rats with standard renal injury does not induce arterial necrosis; rather an ample supply of adrenal steroid of this type seems essential for development of hyperparathyroidism which, in turn, causes vascular injury. Hence a hitherto unknown direct or indirect stimulatory action of mineralocorticoids upon the parathyroid gland must be postulated. Preliminary evidence indicates that, to a certain extent, androgens can enhance this desoxycorticosterone effect, whereas estrogens cannot. In fact, female sex hormone which has been shown to be capable of inhibiting the output of adrenal steroids(6), appears to counteract the development of muscular necrosis.

In line with this observation, the incidence and severity of arteriosclerosis in the young adult rat were found to be substantially higher in male than in female animals. This difference was most pronounced in 2-month-old animals, and gradually decreased with advancing age of the rat. In 2-month-old rats (*not used in this study*), incidence of lesions is around 80% in the male rat and around

TABLE II. Influence of the Parathyroid Gland upon Development of Cardiovascular Necrosis in Nephrectomized Albino Rat. (Male animals, 4-6 mo old.)

Group	Description of procedures	Rat No.	Days of survival post-nephrectomy	Macroscopic pathology Necrosis of	
				Myocardium	Aortic media
A	Control, nephrectomy only	1	3	—	—
		2	2	—	—
		3	4	—	1+
		4	3	4+	2+
		5	3	1+	1+
		6	3	1+	—
		7	3	2+	1+
		8	3	1+	1+
B	Parathyroid extract, 100 I.U. s.c., on 1st and 2nd day post-nephrectomy	9	3	3+	3+
		10	3	4+	1+
		11	3	4+	3+
		12	3	4+	3+
		13	3½	4+	2+
C	Parathyroidectomy, 1 wk prior to nephrectomy	14	3	—	—
		15	3	—	—
		16	4	—	—
		17	4	—	—
		18	5	—	—

Definition of lesions: —, none; 1+, mild, localized; 2+, moderate and more extensive; 3+, severe and widespread; 4+, maximal degree.

50% in the female rat. In the 4-6-month-old group, male rats show an incidence of 95% of mostly severe aortic lesions, compared with an incidence of 70% of moderate to severe lesions in female animals.

In our most recent studies, involving 10 adrenalectomized, 15 parathyroidectomized and 50 nephrectomized rats, the following additional facts were established: (1) It is possible to induce marked hypertension and the characteristic picture of disseminated necrosis in heart, arterial tree and gut in parathyroidectomized rats *not* subjected to standard renal injury, by daily administration of 100 to 200 units of parathyroid extract. These animals may also develop marked nephrocalcinosis. (2) Adrenalectomized rats maintained on saline and not exposed to standard renal injury, manifest invariably pronounced systolic hypertension within 24 hours following injection of 100 units of parathyroid extract with pressures ranging from 160-210 mm Hg. Daily reinjection results in maintenance of hypertension except for a transient marked dip in blood pressure following each administration and lasting about 6 hours. (3) Cardiovascular necrosis and hypertension can likewise be produced in the rat by nephrectomy (Table II, Group A), which speaks against the importance of a renal factor in their development. The organic changes are closely similar to those induced by obstructive nephropathy and not unlike the pathologic-anatomical picture observed by Grollman and associates(7) and others in renoprival animals. (4) Severity of cardiovascular necrosis and hypertension produced by nephrectomy can be substantially enhanced by administration of parathyroid extract (100 units) on first and second day following removal of kidneys (Table II, Group B). (5) When parathyroprive rats are nephrectomized, they do not develop any lesions in the myocardium or the arterial tree recognizable under magnifying glass, although they seem to survive somewhat longer than rats with intact parathyroid glands (Table II, Group C).†

Comments. The concept of the mechanism leading to cardiovascular necrosis and calcification which emerges from our studies is briefly as follows: The "stress" of obstructive nephropathy causes adrenal cortical hypertrophy. Increased output of desoxycorticosterone-like hormones probably triggered primarily by severe derangement of mineral metabolism, results in pronounced stimulation of the parathyroid glands. Autointoxication with parathyroid hormone, in turn, is responsible for the picture of disseminated cardiovascular necrosis and calcification and substantially enhances renal injury (nephrocalcinosis). Concomitant "renal" hypertension probably contributes to the development of vascular lesions. In the light of this concept, it is understandable that mechanisms or procedures which tend to interfere with the availability of the desoxycorticosterone-like steroids, may result in a diminution of the extent and incidence of cardiovascular necrosis, whereas an increase in supply of such steroids may have the opposite effect.

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† (The nephrectomy studies, exemplified in Table II, were carried out in cooperation with Dr. Isabelle Wajda. The results have since been fully duplicated and will be published elsewhere in detail.)

These animal-experimental findings lead us to believe that in human disease as well the parathyroid gland may play a more important role in pathogenesis especially of renogenic cardiovascular necrosis and hypertension than is commonly assumed. For example, it is well known that rapidly progressive malignant hypertension, usually distinguished by extensive medionecrosis of small arteries, is often dramatically arrested by adrenalectomy(8,9). Credit customarily goes to removal of excess mineralo-corticoids and the consequent decrease of hypertension. The possible presence of a hyperfunctioning parathyroid gland is usually considered disproven by the finding of normal blood calcium levels, although it should be realized that, in subacute and chronic phases of clinical as well as experimental hyperparathyroidism, blood calcium levels may return to within the normal range. Moreover, one cannot exclude a possible inhibitory effect of adrenalectomy upon output of a specific vasoactive parathyroid factor, claimed to be separable from the calcium mobilizing activity of parathyroid extract(10) and responsible for both spasm and necrosis of muscles. Such a vasoactive principle may, in fact, represent the long sought extrarenal pressor principle held responsible for both renal and renoprival hypertension and known to be dependent upon adequate adrenal cortical activity(11). Finally, presence of anatomically normal parathyroid glands should not be relied upon as evidence against hyperfunction since, under physiological conditions, these glands were shown to operate only at a small fraction (5-25%) of their full capacity(12). This wide margin of safety allows for excess secretion without alteration of anatomical structure(13). It is conceivable, therefore, that subtotal parathyroidectomy may be more effective than the far more radical adrenalectomy in arresting rapidly progressive sequellae of severe renogenic hypertension.

Our data do not support the time-honored contention that in hyperparathyroidism, calcium deposition occurs in *normal* tissue, so-called "true metastatic calcification" in contradistinction to dystrophic calcification(14), since in our experimental animals calcium imbibition was invariably preceded by disseminated necrosis. Autopsies performed at predetermined stages of the experimental disease proved beyond doubt that areas of necrosis were those which later became calcified. It is of special interest in this connection that in a recently reported post mortem examination of a patient who died from renogenic hyperparathyroidism, the myocardium was found to contain foci of fresh necrosis, a fact which in the authors' view was the reason for extreme severity of "metastatic calcification" (15). Significantly they state that the cause for necrosis is obscure. In the more chronic phase of hyperparathyroidism when healing predominates, especially following various therapeutic efforts, necrosis may be absent, so that foci of calcification appear embedded in otherwise normal tissue. This is the very situation most commonly encountered at autopsy in human beings. It is probably responsible for the concept of "metastatic calcification."

Finally, it would appear in the light of our findings that a number of experimental lesions ascribed in particular to direct effects of desoxycorticosterone and saline(16) or a combination of these with renal insufficiency(17) may deserve reevaluation.

Summary. 1. Pathogenesis of severe arteriosclerosis and disseminated myocardial necrosis, induced in the albino rat by standard renal injury, was investigated. These cardiovascular lesions are due to autointoxication with parathyroid hormone, and this hormone apparently contains an important hypertensive factor. 2. Evidence was presented that excess production of parathyroid hormone, as a consequence of renal dysfunctions, is mediated through the adrenal cortex and that mineralocorticoids are essential for direct or indirect activation of the parathyroid gland. To some degree, androgens can enhance the mineralocorticoid effect, whereas estrogens seem to inhibit emergence of muscular necrosis. The incidence of cardiovascular injury in intact and adrenalectomized rats was found to be substantially higher in male than in female animals. 3. It was shown further that, in the absence of parathyroid glands, mineralocorticoids in excessive dosage fail to produce cardiovascular necrosis, whereas excess parathyroid hormone assures emergence of typical lesions in complete absence of mineralocorticoids. In fact, by providing abundant supplies of parathyroid extract, cardiovascular necrosis was induced without renal injury and even in absence of kidneys. 4. Implications of these findings upon the concept of "metastatic calcification" and upon pathogenesis and therapy of renogenic cardiovascular necrosis and hypertension in human beings are discussed. It is suggested that subtotal parathyroidectomy be tried in preference to adrenalectomy in attempts to arrest rapidly progressive sequellae of malignant hypertension.

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1. Lehr, D., and Martin, C., *Endocrinology*, 1956, v59, 273.

2. Lehr, D., and Churg, J., *J. Mt. Sinai Hosp*, 1952, v92, 32.

3. Lehr, D., unpublished data, 1953.

4. Rappaport, F., *Rapid Microchemical Methods of Blood and CSF Examinations*, Grune & Stratton, New York, 1941, p116.

5. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, v66, 375.

6. Vogt, M., *J. Physiol.*, 1955, v130, 601.

7. Muirhead, E. E., Turner, L. B., and Grollman, A., *A.M.A. Arch. Path.*, 1951, v51, 575.

8. Green, D. M., Nelson, J. N., Dodds, G. A., and Smalley, R. E., *J.A.M.A.*, 1950, v144, 439.

9. Thorn, G. W., Harrison, J. H., Merrill, J. P., Criscitiello, M. G., Frawley, T. F., and Finkenstaedt, J. T., *Ann. Int. Med.*, 1952, v37, 972.

10. Handler, P., and Cohn, D. V., *Am. J. Physiol.*, 1952, v169, 188.

11. Wilson, C., and Hedingham, J. M., *Acta Med. Scand.*, 1956, v154, 86.

12. Rosof, J. A., *J. Exp. Zool.*, 1934, v68, 121.

13. Greep, R. O., *Histology*, Blakiston Co., New York, 1954, p822.

14. Barr, D., *Physiol. Rev.*, 1932, v12, 593.

15. Crawford, R. L., and La Zerte, G. D., *Arch. Int. Med.*, 1955, v96, 818.

16. Selye, H., Hall, G. E., and Rowley, E. M., *Canad. M. A. J.*, 1943, v49, 88.

17. Selye, H., and Pentz, E. I., *ibid.*, 1943, v49, 264.

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Gastric Iodide and Chloride Clearances in Dogs.* (22835)

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Inorganic iodide is concentrated in gastric juice of experimental animals(1-4) and human beings(5-8) as effectively as in the thyroid gland(9). Investigation into the mechanisms was complicated by injection of sodium iodide(1,2) which partially interfered with the iodide-concentrating process(1). Several authors(1,2) have suggested that iodide might replace chloride in gastric juice but investigations regarding secretory drugs(3,4) have not included quantitative chloride and iodide data that would test the possible interchange of iodide and chloride.

Plasma clearances of iodide by the stomach have been determined in patients(8) and in rats(10). The iodide and chloride clearances were considered satisfactory quantitative indices of gastric secretory mechanisms. The present investigations were undertaken to compare these functions under the influence of substances known to alter the iodide trap of the thyroid gland(11). It was considered that clearance studies might give a basis for comparison of iodide and chloride secretory mechanisms of the stomach and some comparison of the gastric and thyroid iodine trapping mechanisms.

Methods. Acute experiments with Nembutal anesthesia: Mongrel dogs were fasted 6-18 hours before the experiment. They were lightly anesthetized by intraperitoneal injection of 1 cc 5.5% Nembutal per 5 lb. The femoral veins were exposed on both sides, one for injections and the other for blood sampling. The stomach was exposed by a midline incision, the esophagus tied with strong cord, and the pylorus cannulated by tying into place a thick rubber tube introduced into the stomach through the duodenum. Then 15 to 90 μc of I^{131} were injected intravenously. After a 10-30 minute period for mixing, the stomach was washed with approximately one liter of warm water in 3-4 portions and then returned to its normal position in the abdomen. After 7.5 minutes a blood sample was taken and after 7.5 additional minutes the stomach's secretions were collected by washing with a liter of water in 3 or 4 equal portions. This procedure was repeated in order to have 2 control collections after which the animal was injected with either Histamine (3 mg), NaSCN (75 mg/kg), NaI (60 mg/kg), or acetazoleamide (Diamox, .5-1 g). After 10-30 minutes, the stomach was washed and 2 collections of gastric secretions made and a blood sample taken at the mid-time of each. The gastric secretions and blood were analyzed for I^{131} by scintillation counting. Chloride was determined mercurimetrically(12). Gastric iodide and chloride clearances were determined as follows: $\frac{\text{Gastric } I^{131} \text{ or chloride/min.}}{\text{Plasma } I^{131} \text{ or chloride/cc}}$. *Chronic experiments on unanesthetized dogs with Heidenhain or innervated total gastric pouch:* The dogs were fasted 18 hours preceding the experiment, and allowed water *ad libitum.* A venous blood sample was drawn preceding the experiment and analyzed for radioactivity to determine residual activity left from previous experiments. If activity was found, PBI^{131} was determined; this never exceeded 3% of the total plasma I^{131}. Ten to 40 μc of I^{131} were injected into the left leg vein. After a 30 minute period for mixing and equilibration, the pouch was washed with 200 to 300 cc of warm water, using 60 cc washing. Seven to 8 minutes later a blood sample was taken from the right leg and at 15 minutes a gastric collection was made by washing the stomach with 200 cc of water in 60 cc portions. After the 2 control collections, the dogs were injected with Histamine (3 mg), NaSCN (75 mg/kg), $NaClO_4$ (15 mg/kg), NaI (60-100 mg/kg) or reserpine (.25 mg/

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† USPHS Medical Student Fellow.

TABLE I. Gastric Clearance of Iodide and Chloride.

Procedure	No. of exp.	Gastric clearance of plasma, cc/min.	
		Iodide	Chloride
	Acute exp., Nembutal anesthesia		
Control	6 (3 dogs)	4.7 (2.4-7.5)*	.40 (.17-.67)
Sodium iodide, 60 mg/kg		.53 (.28-1.0)	.35 (.13-.61)
Control	4 (2 ")	3.4 (3.0-4.3)	.46 (.33-.74)
Thiocyanate, 75 "		.39 (.32-.41)	.26 (.20-.30)
Control	8 (4 ")	7.3 (4.3-10.2)	.27 (.12-.46)
Histamine, .1-.14 "		9.9 (7.3-12)	.66 (.24-1.3)
Control	6 (3 ")	4.2 (3.1-5.3)	.26 (.16-.32)
Diamox, .5-1.0 g		4.9 (2.9-6.4)	.24 (.15-.30)
	Unanesthetized female Heidenhain pouch dog		
Control	2	5.4 (4.8-6.0)	.27 (.21-.34)
Sodium iodide, 60 mg/kg		.70 (.60-.80)	.12 (.10-.14)
Control	2	4.4 (4.1-4.7)	.11 (.10-.12)
Thiocyanate, 75 "		.25 (.25-.45)	.12 (.10-.15)
Control	2	1.5 (1.3-1.6)	.12 (.11-.14)
$NaClO_4$, 15 "		.11 (—)	.12 (.11-.12)
Control	2	4.5 (4.4-4.5)	.10 (—)
Histamine, 3 mg		12 (11-14)	1.2 (.94-1.5)
Control	2	5.2 (4.8-5.7)	.25 (.24-.26)
Serpasil, 2.5 "		12 (9.5-14)	.87 (.57-1.2)
	Male Heidenhain pouch dog		
Control	2	5.4 (4.6-6.3)	.10 (—)
Na I, 100 mg/kg		.40 (.37-.42)	.08 (—)
Control	2	2.8 (2.6-3.0)	.08 (—)
NaSCN, 75 "		.32 (—)	.08 (—)
Control	2	3.2 (3.1-3.3)	.10 (.09-.11)
$NaClO_4$, 15 "		.13 (.12-.14)	.11 (.10-.12)
Control	2	5.5 (4.3-6.7)	.09 (—)
Histamine, 3 mg		25 (23-27)	1.7 (1.5-1.9)
	Male total innervated pouch dog		
Control	2	6.7 (6.4-7.0)	.19 (.15-.23)
NaI, 60 mg/kg		1.4 (1.1-1.6)	.15 (.12-.18)
Control	2	12 (9.4-14)	.27 (.25-.29)
NaI, 100 "		.90 (—)	.14 (.13-.15)
Control	4	4.3 (2.9-6.2)	.17 (.15-.19)
NaSCN, 75 "		.7 (.37-.80)	.16 (.09-.23)
Control	4	4.8 (3.4-6.8)	.20 (.12-.34)
$NaClO_4$, 15 "		.36 (.27-.54)	.21 (.13-.33)
Control	4	1.5 (1.0-2.7)	.24 (.17-.42)
Histamine, 3 mg		11 (9.0-13)	2.6 (1.7-3.3)

* Range; if no range stated there were 2 identical values.

kg). After a 30 minute period for mixing and equilibration, the pouch was washed and 2 15-minute experimental gastric collections made. Blood samples were taken at the mid-time of each. These specimens were analyzed for gastric clearance of chloride and I^{131} in the same manner as the samples from the acute experiments.

Results. Table I shows the control gastric I^{131} clearances varied among dogs and on the same dog from day to day. Control gastric I^{131} clearances varied from 1.5 to 11.7 cc plasma/min. The control gastric chloride clearances were 0.1 to 0.46 cc plasma/min., *i.e.*, 2% to 12% as great as the control iodide clearances. NaI administration depressed I^{131}

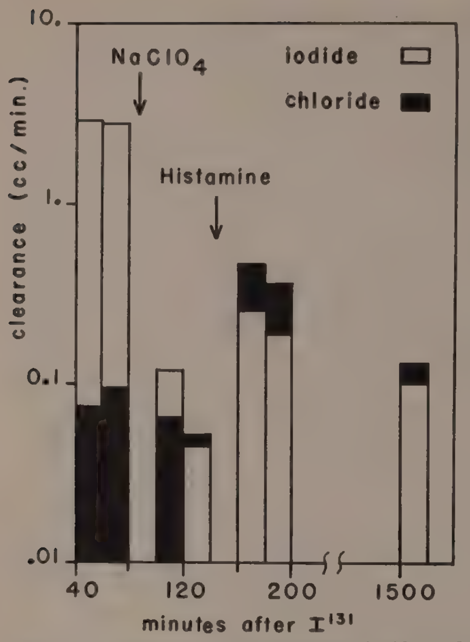

FIG. 1. Alterations in simultaneous iodide and chloride clearances of Heidenhain pouches. Each value represents an avg from 2 dogs. After each drug ($NaClO_4$ and histamine) one 20-min. collection was discarded without analysis.

clearance until it approached the chloride clearance. NaI did not cause as great a decrease in chloride clearance but there was the suggestion of a consistent reduction following iodide administration. NaSCN reduced gastric I^{131} clearance similar to NaI with no effect on chloride clearance except in the anesthetized dogs (Table I). $NaClO_4$ in ⅕ the dose of NaSCN produced the most severe reduction in I^{131} clearance which fell consistently to the level of chloride clearance. In these doses $NaClO_4$ produced no effect on chloride secretion. A single dose of perchlorate continued to exert maximum depression of gastric iodide clearance for more than 2 days in 3 dogs so studied.

Histamine produced a 10 to 18 fold increase in gastric chloride clearance in conscious dogs and 3 to 7 fold increase in the gastric I^{131} clearance.

In 2 dogs (Fig. 1) histamine was injected after demonstration of the $NaClO_4$ effect. The gastric chloride and iodide clearances then showed similar histamine responses.

Serpasil, used in 2 experiments on one dog, doubled the gastric I^{131} clearance and tripled the gastric chloride clearance. The Serpasil depressed the animal, and death occurred 3 days after the experiment. Diamox had no effect on basal chloride or I^{131} secretion.

Discussion. Apparently this is the first quantitative determination of the gastric I^{131} clearance in dogs. The results show: (1) those substances which greatly reduced the secretion of iodide by the gastric mucosa had little or no effect on the basal secretion of chloride, (2) histamine and reserpine produced a greater percent rise in the gastric chloride clearance than in iodide clearance, (3) the basal chloride clearance was only 2 to 12% of the basal iodide clearance, (4) when iodide secretion was blocked by a substance known to inhibit the thyroidal iodide trap, the gastric iodide clearance approached or reached the basal chloride clearance values. These data suggest that there are possibly two mechanisms for the secretion of iodide by the stomach, one that specifically traps iodide and rapidly secretes it, another non-specific mechanism in which iodide and chloride are interchangeable. This non-specific process was stimulated by histamine and not readily blocked. The specific process was effectively inhibited by elevated plasma iodide and by NaSCN or $NaClO_4$, similar to the iodide trap of the thyroid gland. Previous investigators who administered NaI probably blocked this specific iodide trap. The effect of $NaClO_4$, NaI, and NaSCN on the I^{131} clearance is possibly due to the anions of these salts combining with a specific system necessary for the transfer of iodide across the gastric cell. For normal function, this specific iodide trap may require an unsaturated carrier.

Histamine in absence of perchlorate (Table I) increased iodide clearance an average of 4 fold in unanesthetized dogs. Histamine in presence of perchlorate (Fig. 1) resulted in a similar (5 fold) increase in iodide clearance. Comparison of absolute results in gastric pouch dogs illustrates a dissimilarity between effects of histamine before and after perchlorate. Before $NaClO_4$ injection, histamine increased the iodide clearance an average of 12 cc/min. but after $NaClO_4$, the increase was less than 1 cc/min. Therefore, the effects of histamine before and after perchlorate were relatively similar but absolutely quite different. This suggests that histamine had some effect upon the specific iodide trap of the stomach.

Summary. Iodide and chloride clearances were determined in anesthetized and unanesthetized dogs. The control gastric iodide clearance was 10-50 times greater than the chloride clearance. The iodide clearance could be 95% suppressed by anions known to block the thyroid iodide trap but chloride clearance was practically unchanged. In the presence of the blocking agent, gastric iodide and chloride clearances were similar. These data suggest at least 2 mechanisms for gastric secretion of iodide: (a) a specific iodide secretory system and (b) a non-specific mechanism in common with chloride secretion which continues to function after blockade of (a).

1. Davenport, H. W., *Gastroenterology*, 1943, v1, 1055.

2. Lagergreen, B. R., *ibid.*, 1950, v14, 558.

3. Mason, E. E., and Bloch, H. S., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 488.

4. Reugamer, W. R., *Arch. Biochem. and Biophysics*, 1953, v47, 119.

5. Honour, A. J., Myant, N. B., and Rowlands, E. N., *Clin. Sci.*, 1952, v11, 447.

6. Myant, N. B., *et al.*, *ibid.*, 1950, v9, 405.

7. Schiff, L., *et al.*, *J. Clin. Invest.*, 1947, v26, 1196.

8. Oeff, K., Kessel, M., and Krentz, K., *Klin. Wschr.*, 1955, v33, 59.

9. VanderLaan, J. E., and VanderLaan, W. P., *Endocrinology*, 1947, v40, 403.

10. Brown, Josiah, *ibid.*, 1956, v58, 69.

11. Wyngaarden, J. B., Wright, B., and Ways, P., *Endocrinology*, 1952, v50, 537.

12. Hawk, P. B., Oser, B. L., and Sommerson, W. H., *Practical Physiological Chemistry*, P. Blakiston's Son and Co., Philadelphia, 1954, 13 ed., p626.

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Frequency of Abortive Parthenogenesis in Domestic Turkey.*† (22836)

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Abortive parthenogenesis in the chicken has been demonstrated to occur in eggs laid by virgin hens(1,2). Parthenogenesis also occurs in the turkey(3-5) and most of this development is of abnormal type. The available cytological evidence(6) is that nuclei of such parthenogenetically developing tissue are diploid. Kosin(7,8), in the absence of evidence to the contrary, ruled out parthenogenesis as a major factor responsible for the presence of aggregates of "moribund" cells observed in germ discs of apparently infertile eggs from mated hens. Following the first report by Olsen and Marsden(3) on the occurrence of parthenogenetic development in the turkey, a more intensive study of the matter was undertaken.

Materials and methods. The period reported here covers 1952-1956. The eggs were produced by the College strain of Broad Breasted Bronze hens and by U. S. D. A. strain of Beltsville Small White hens. The hens had been separated from males since 10-14 weeks of age. Upon separation, the birds were brought into completely enclosed pens where they remained for the entire observation period. Because the pens lacked windows, a low intensity artificial light (less than 1-foot candle power) was provided until the birds were 28 weeks old. At that time, the light intensity was increased to more than 5-foot candle power to stimulate oviposition.

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TABLE I. Frequency of Parthenogenetic Development in Broad Breasted Bronze Eggs.

	Unincubated		Incubated for 7 days	
Year	No. eggs	Positives, micro. examinations	No. eggs	Positives, micro. examinations
1954	101*	59 (58.4%)†	522	10 (2.8%)
1955			2594	65 (2.5%)

* These eggs received 10 hr of incubation.
† % of total eggs.

The 1953, 1954 and 1956 egg records were kept on pen-basis, while in 1955 trap nesting was introduced to identify the egg according to hen. Moreover, in the latter case there was an environmental difference: In one pen (No. 71), beginning in November, temperature was maintained at 55° ± 5°F; in the other pen (No. 70) the temperature fluctuated as before. The 1953 study, based on 1952 hatched birds, was designed primarily to develop suitable technics for assessing the prevalence of parthenogenesis in the domestic turkey. In 1954 the study was expanded to include observations on nearly 800 eggs. Some germ discs were studied histocytologically without first being subjected to incubation. Others were incubated for 7 days before being observed for gross signs of development. All 1955 and 1956 eggs were incubated for 7 days prior to examination. Discs which showed any evidence of tissue growth in the germ disc area were preserved for further histo-cytological study.

Results. The 1953 and 1954 material demonstrated that abortive parthenogenetic development occurs in the domestic turkey with higher frequency than that reported for the domestic chicken(1). Study of the 1954 eggs revealed that up to 80% of unincubated eggs laid by virgin turkey hens showed signs of parthenogenetic development. However, only a fraction of the germ discs showing such development on a cellular level, had the capacity to develop further (Table I).

In 1955 there was an indication of a difference in potential to develop parthenogenetically in the two groups subjected to different environments. In pen 70 (no temperature control) 3.5% of the eggs showed development; in pen 71 (temperature control), the corresponding figure was 2.2%. A complicating factor was introduced by the fact that birds in pen 70 developed a type of non-specific respiratory condition early in January, before egg laying began (trap-nesting was started on Jan. 23).

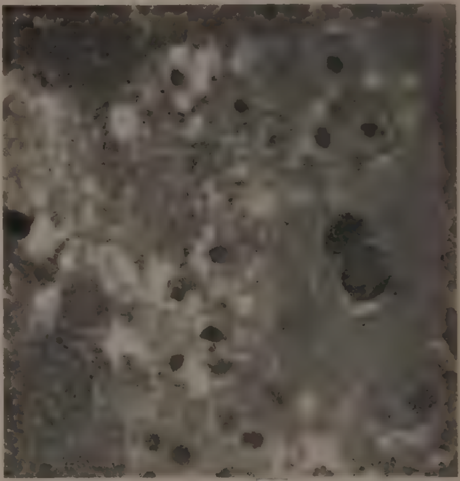

FIG. 1. Example of "single-cluster" type of cell proliferation in unincubated egg laid by virgin turkey hen.

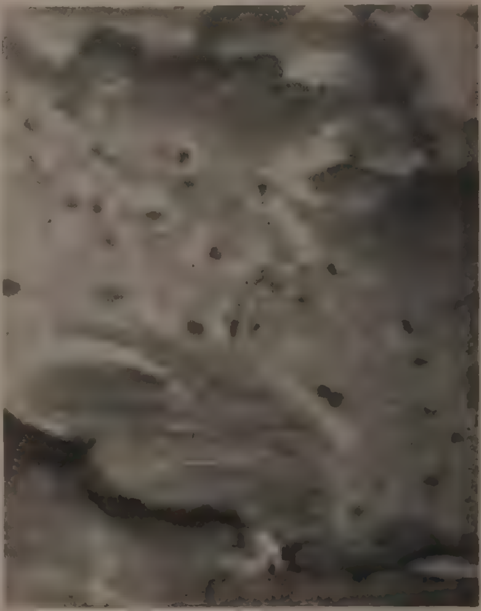

FIG. 2 and 3. A more extreme type of development than shown in Fig. 1, frequently found in unincubated eggs from virgin turkey hens.

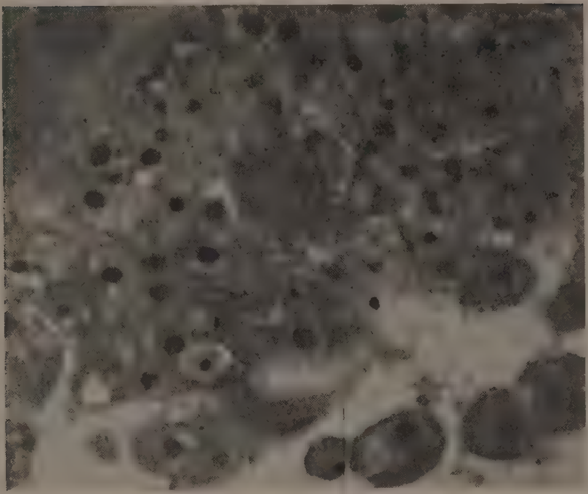

FIG. 3.

The unincubated turkey eggs which showed parthenogenetic development almost invariably were characterized by the presence of a single cluster of cells. In a number of instances, however, more than one such aggregate was observed in the blastodisc. (Fig. 1, 2, and 3.) The nuclei usually showed moderate affinity for basophilic stains, although only a few examples of active cell division have been encountered. This agrees with the observation that only a small portion of such germ discs are capable of developing further. In a great majority of cases, these cell clusters undoubtedly are either moribund cells or those which have recently died.

The observed level of abortive parthenogenesis in the Broad Breasted Bronze stock, as studied in this investigation, was considerably below that reported for Beltsville Small Whites(2,5). To check on this further, 100 eggs laid by virgin Beltsville Small White hens, and 100 eggs laid by mated Beltsville Small White hens of the strain which showed a high level of "positives" in the Beltsville work, were obtained from the Beltsville Research Center in late spring of 1955.‡ These B. S. W. eggs from virgin hens were incubated for 7 days and examined for evidence of "embryonic" growth. The eggs from the mated hens were used to provide an experimental flock of virgin Beltsville Small White hens raised under Pullman conditions.

Table II gives comparative figures on the frequency of parthenogenetic development in the eggs from 2 locations. There is obviously a genetic difference in the potential of the eggs to show such development. The location *per se* is not a major factor affecting the expression of that potential. Olsen and Marsden(2) have demonstrated that similar differences exist among the turkeys and chickens studied by them.

The fact that abortive parthenogenesis is of such common occurrence in turkeys poses certain problems. For example: Is the poten-

TABLE II. Population Differences in Frequency of Parthenogenesis following 7 Days of Incubation.

Year	Variety	No. of eggs	Positives, gross examination
1955	B. S. W.*	100	18.0
	B. B. B.	2954	2.9
1956	B. S. W.†	314	31.2
	B. B. B.	214	4.2

* Based on eggs obtained from U. S. D. A.

† Based on eggs laid by Beltsville Small White hens hatched in Pullman in 1955. 1956 data for both varieties include information up to June 6.

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‡ The authors wish to acknowledge the cooperation of Drs. T. C. Byerly and W. A. Brant of the Agriculture Research Service, USDA, in providing these eggs.

tial for parthenogenetic development expressed to a greater or lesser degree in flocks of mated birds? Obviously, the answer to this question has a direct bearing upon the interpretation placed on finding cell colonies in so-called "infertile" eggs. Thus far, these usually have been considered to be instances of early embryonic mortality(8). Which of these "infertile" eggs from mated hens are developing parthenogenetically and which are cases of delayed development following fertilization? Olsen and Marsden(9) suggested that those turkey eggs from mated hens which show development only after 72 hours of incubation may be instances of parthenogenesis. Krizenecky *et al.*(10) approached the problem of morphological differences between infertile and non-fertile (from virgin hen) chicken eggs on the basis of the appearance of the germ disc. Unpublished data from this laboratory indicate that there may be similar differences in the turkey.

Another question is: Can the predisposition to parthenogenetic development serve as a deterrent to normal fertilization? It is conceivable that eggs possessing a high potential for such development would be less capable of being fertilized.

Summary. Evidence has been presented that: (1) Frequency of abortive parthenogenesis in the Broad Breasted Bronze turkey is as high as 80% when based on unincubated eggs studied histo-cytologically. (2) There are varietal differences which affect the frequency of occurrence of this type of development. Possibly, environmental differences also affect the frequency of abortive parthenogenesis.

1. Kosin, I. L., *Anat. Rec.*, 1945, v91, 245.
2. Olsen, M. W., and Marsden, S. J., *Poultry Sci*, 1954, v33, 1074. (Abstract).
3. ———, PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 638.
4. ———, *J. Exp. Zool.*, 1954, v126, 337.
5. ———, *Science*, 1954, v120, 545.
6. Yao, T. S., and Olsen, M. W., *J. Hered.*, 1955, v46, 133.
7. Kosin, I. L., *Proc. 8th World's Poul. Congress*, 1948, v1, 345.
8. ———, *Poultry Sci.*, 1951, v30, 805.
9. Olsen, M. W., and Marsden, S. J., *ibid.*, 1954, v33, 1146.
10. Krizenecky, J., Sajner, J., Orel, V., and Musil, F., Detection of fertilized, infertile and nonfertilized eggs in biological control of incubation and in testing of commercial eggs. *Ministerstvo Potravinarskeho Promyslu, Prague*, 1955.

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Effect of Anesthetics and Collection Time on Corticosteroid Secretion By Rat Adrenal.* (22837)

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Measurement of corticosteroids in the adrenal vein effluent of the rat has been described by Bush(1), Singer and Stack-Dunne (2) and Rosenman *et al.*(3). However, no attempt has been made to correlate secretion rate under these circumstances with time of collection of effluent and the nature of anesthetic employed. Several lines of evidence suggest that ether anesthesia is more stressful than Nembutal (pentobarbital sodium) and evokes a more profound pituitary-adrenocortical response than the latter(4-10).

The present observations support this conclusion and demonstrate that ether, but not Nembutal, anesthesia results in a marked early elevation of corticosterone in the ad-

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renal venous effluent of the rat.

TABLE I. Photometric Analysis of Corticosterone Standards.*

Corticosterone, μg	No. of analyses	Optical density[†] (565 mμ)	Error, %[‡]	K[§]
4	15	.02 ± .002	30	.50
6	9	.03 ± .002	20	"
8	14	.04 ± .002	20	"
10	10	.05 ± .003	18	"
16	15	.08 ± .004	21	"
24	24	.12 ± .004	17	"
32	17	.17 ± .007	17	.53
40	10	.20 ± .010	17	.50

* Corticosterone was chromatographed, stained with Tetrazolium Blue and eluted with ethyl acetate-methanol (see text).

[†] Mean ± stand. error.

[‡] Stand. dev./mean.

[§] Mean optical density/μg corticosterone.

Methods. Adult, male Sprague-Dawley rats from our breeding colony were employed and maintained *ad libitum* on Purina laboratory chow and tap water. They weighed 300 to 500 g. Cannulation of the left renal vein was performed under ether or Nembutal anesthesia. Complete anesthesia was usually attained within 1 to 2 minutes with ether, and in approximately 15 minutes with Nembutal (50 mg/kg injected intraperitoneally). The technique of cannulation was similar to that described by Bush(1), as modified by Singer and Stack-Dunne(2), and was accomplished within 10 to 15 minutes after laparotomy. Heparin was used as an anti-coagulant. A series of timed collections was made of the left adrenal venous effluent in individual animals. The amount of blood collected was usually 2 to 4 ml for each time interval. Corticosteroids were extracted from blood samples by the method of Bush(1), as modified by Singer and Stack-Dunne(2). The extracts were then chromatographed on Whatman No. 1 filter paper employing toluene: methanol:water (4:3:1) system described by Bush(1). Known amounts of corticosterone and cortisol[†] were run as markers with each determination. Chromatographic development was allowed to proceed for 100 to 150 minutes at room temperature. Consistent results were obtained without temperature control. A complete change of the solvent phases within the chromatography cylinder was made only once every 3 to 4 months. Resolved steroids were located by spraying the air-dried filter paper sheets with alkaline Tetrazolium Blue[‡] solution(11). The bulk of the corticosteroids in rat adrenal effluent invariably migrated in the corticosterone zone(1-3). Rarely, a trace of cortisol appeared as well(1). Elution of the stained steroids, as described by Touchston and Hsu(11), was carried out by immersing the dried chromatograms in distilled water for 3 minutes, followed by partial drying at room temperature. Sections of damp filter paper containing stained steroid were cut into small fragments and eluted twice with 5 ml of ethyl acetate:methanol (7:3). Each elution was for 10-15 minutes, with occasional shaking. Appropriate blank determinations were performed concurrently on unstained portions of the same chromatogram. These blanks usually had an absorbance of .02-.03, paralleling the average reading of .0269 reported by Touchston and Hsu(1). Photometric estimation of the dye eluted from the steroid-containing and control portions of chromatogram was made at 565 mμ in the Coleman Junior Spectrophotometer against a solvent blank set at zero density. Except for the 0 to 10 minute blood collections under Nembutal anesthesia, all density readings were equal to or greater than 0.05. The effective instrument error in this range is less than 10%. In the 0 to 10 minute Nembutal series only 1 reading was in this range. The value recorded for corticosterone secretion in this series (Table II) must therefore be considered maximal. *Quantitative chromatography* of pure corticosterone in amounts similar to those present in blood extracts yielded the results shown in Table I. The optical densities of the eluted dye followed Beer's Law (K = 0.50) over the range of 4 to 40 μg of corticosterone. The error inherent in these determinations averaged about 20% for concentrations of this steroid above 6 μg. In addition, recovery of corticosterone added to 2 ml of peripheral rat blood,

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[†] Samples of pure corticosterone and cortisol were generously made available by Dr. Elmer Alpert, Merck and Co., Rahway, N. J.

[‡] Nutritional Biochemicals Corp., Cleveland, O.

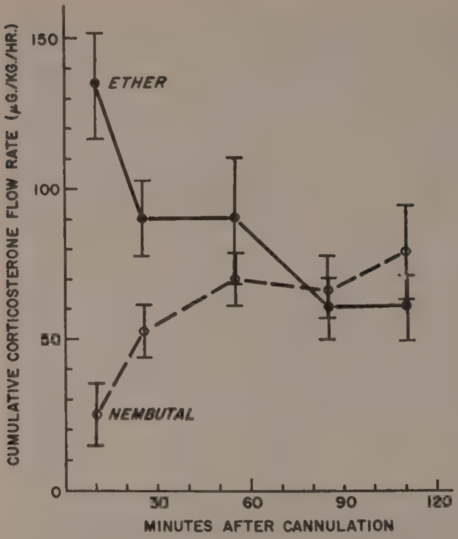

FIG. 1. Influence of anesthetic and collection time on cumulative corticosterone secretion rates of rat adrenal. Each point represents mean of 6 to 16 determinations, each on a different animal; stand. error is also shown.

and processed as described above, averaged 98% (90 to 104%) in 4 experiments with 50 µg of added steroid and 102% (88 to 144%) in 3 experiments with 10 µg of added steroid.

Results. The influence of time of collection and type of anesthetic on the amount of corticosterone appearing in the adrenal venous effluent of the rat following cannulation is depicted in Table II. Ether anesthesia resulted in elevated values for corticosterone concentration and corticosterone flow rate in the first 10 minutes of collection, as compared with corresponding values obtained after Nembutal anesthesia. This early difference was not evidenced in later collections (10 to 25 minutes and 25 to 85 minutes). However, cumulative values for rate of corticosterone release from the time of cannulation to the end of each collection period revealed that the differential effect of ether and Nembutal was apparent in collections made for 25 minutes or less, but not thereafter (Fig. 1).

It was not possible in the present investigations to dissociate the effects of anesthesia on corticosteroid release from those of the operative and collection procedures. It would appear likely, however, that the elevated early values observed after ether anesthesia were due, in part, to pituitary-adrenal activation associated with the administration of this anesthetic. A similar conclusion may be drawn from recent work on the effect of ether anesthesia on blood ACTH(4) and adrenal ascorbic acid levels(5) in the rat and on corticosteroid release by the dog adrenal (6). The use of ether as an anesthetic in studies on pituitary-adrenocortical function would appear to be contraindicated.

It is not certain whether the lower initial values for corticosterone release obtained after Nembutal anesthesia reflect the basal non-stressed corticosteroid output of the rat adrenal or partial inhibition of the early response to the stress of laparotomy. The latter possibility is supported by the observations of Sayers and Sayers(7), Ronzoni(8) and Cronheim and Hyder(9) on the inhibition by Nembutal of the adrenal ascorbic acid response to stress in the rat.

The similarity of corticosterone output values obtained during the later collection periods (Table II) under either Nembutal or ether anesthesia suggests that these later values represent the response of the rat adrenal cortex to the continued stresses of cannulation and blood loss. The maximal corticosterone concentrations observed in the present studies were 6.7 µg per ml of effluent with ether anesthesia and 7.0 µg per ml with Nembutal during the final collection period (25 to 85 minutes following cannulation).

The potentiating action of ether and the depressing effect of Nembutal on corticosterone secretion immediately following cannulation may be presumed to have been mediated via the hypothalamic-hypophyseal system. A central action of ether is supported by the observation of Sydnor and Sayers(4) that this anesthetic resulted in a rapid elevation of blood ACTH in the rat. However, a more direct effect of ether on the pituitary-adrenal system is not eliminated by these studies. The report by Cronheim and Hyder (9) that complete anesthesia with Nembutal blocked the response of the pituitary-adreno-

TABLE II. Effect of Anesthetic and Collection Time on Corticosterone Secretion by Rat Adrenal.

		Ether			Nembutal			
	Collection time, min.*	No. of rats	Vol of blood, ml†	Corticosterone secretion†	No. of rats	Vol of blood, ml†	Corticosterone secretion†	P value‡
Corticosterone flow rate (μg/kg/hr)	0-10	7	2.6 ± .4	136 ± 20	7	3.0 ± .8	25 ± 12	<.001
	10-25	6	2.0 ± .3	62 ± 10	5	1.8 ± .2	44 ± 24	.5
	25-85	11	3.8 ± .7	72 ± 15	16	4.4 ± .3	76 ± 12	.8
Corticosterone conc. (μg/ml)	0-10	7	2.6 ± .4	4.1 ± 1.0	7	3.0 ± .8	.9 ± .5	<.001
	10-25	6	2.0 ± .3	3.5 ± 1.2	5	1.8 ± .2	2.8 ± 1.3	.8
	25-85	11	3.8 ± .7	6.7 ± .8	16	4.4 ± .3	7.0 ± .9	.9

* Mean collection time. Collection times designated as 25 min. actually varied from 20 to 30 min.; those designated as 85 min. varied from 60 to 115 min.

† Values expressed as mean ± stand. error.

‡ Significance of differences between corticosterone secretion in Nembutalized *vs* etherized animals.

cortical system in the rat to salicylic acid suggests that this anesthetic exerts its primary action on the hypothalamus. The role of the hypothalamus in the activation of the pituitary-adrenal system is well established(12).

Cumulative corticosterone flow rates in the adrenal venous effluent of the rat obtained in the present study were somewhat lower than those reported by Singer and Stack-Dunne (2) and Rosenman *et al.*(3) under similar conditions. Variations in technic and age and strain of animals may be responsible for these differences. The considerably higher values reported by Bush(1) have been attributed, in part, to the administration of exogenous ACTH to his experimental animals. However, judging from the present investigations, the short collection time (10 to 20 minutes) under ether anesthesia may also have contributed to these high values.

Summary. Secretion of corticosterone into the adrenal venous effluent of the rat was markedly influenced during the first 10 minutes of collection by the nature of the anesthetic employed for cannulation procedure. Ether anesthesia resulted in a significant elevation of corticosterone concentration and corticosterone flow rate during this period as compared with the values observed when Nembutal was used. This difference was eradicated during subsequent collection periods of 10 to 25 minutes and 25 to 85 minutes following cannulation. The relationship of these findings to the possible activation by ether and inhibition by Nembutal of the pituitary-adrenocortical system is discussed.

1. Bush, I. E., *J. Endocrinol.*, 1953, v9, 95.
2. Singer, B., and Stack-Dunne, M. P., *ibid.*, 1955, v12, 130.
3. Rosenman, R. H., St. George, S., Freed, S. C., and Smith, M. K., *J. Clin. Invest.*, 1955, v34, 1726.
4. Sydnor, K. L., and Sayers, G., *Endocrinology*, 1954, v55, 621.
5. Munson, P., and Briggs, N., *Recent Progress in Hormone Research*, 1955, v11, 83.
6. Nelson, D. H., Egdahl, R. H., and Hume, D. M., *Endocrinology*, 1956, v58, 309.
7. Sayers, G., and Sayers, M. A., *Recent Progress in Hormone Research*, 1948, v2, 81.
8. Ronzoni, E., *Am. J. Physiol.*, 1950, v160, 499.
9. Cronheim, G., and Hyder, N., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 409.
10. Semonsen, C. P., and Sawyer, C. H., *Am. J. Physiol.*, 1954, v177, 405.
11. Touchston, J. C., and Hsu, C.-T., *Analytical Chem.*, 1955, v27, 1517.
12. Long, C. N. H., *Ann. Rev. Physiol.*, 1956, v18, 409.

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Conversion and Maintenance of *Histoplasma capsulatum* in Tissue Culture. (22838)

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The conversion of the diphasic fungus, *Histoplasma capsulatum,* to its tissue phase, has always been difficult, in our experience. However, many artificial media, such as a modification of Francis' cystine glucose blood agar (1), brain-heart infusion glucose blood agar and Salvin's synthetic medium(2), have been successfully used by many investigators. In recent years Kurung's egg(3), and Littman's liver-spleen media(4) were developed more or less specifically for the conversion and maintenance of *H. capsulatum* in its yeast phase. In addition to the artificial media Randall and his co-workers have advocated the use of tissue culture methods for the study of this fungus. They were successful in the *in vitro* cultivation of the tissue phase in media containing horse tissue, chicken fibroblast-like cells and the Earl's strain "L" mouse cells (5-7).

In preparation for studies on efficacy of antifungal agents it became evident that a more suitable, rapid and consistent method of obtaining the tissue phase of *H. capsulatum* had to be found. Therefore, the HeLa tissue culture method was explored in an effort to determine the efficiency of another animal material in which to study the fungus. Studies were made of the conversion and maintenance of the fungus with and without HeLa cells and its maintenance with and without rotation of the culture drum.

Methods and materials. The 13 isolates of *H. capsulatum* used in these studies were obtained from recent human cases. They were isolated from clinical specimens shipped to this laboratory. HeLa cells were cultivated in 250 ml bottles employing a medium composed of 20% human serum, 8% of a 1% yeast extract, 1% of a 20% glucose solution, 68% Hank's balanced salt solution (B.S.S.), 3% of a solution of 2.8% sodium bicarbonate and 0.001% phenol red. One hundred units of penicillin, 100 μg of streptomycin, and 0.2 μg of butyl parahydroxybenzoate were added to each ml of medium. The cells were trypsinized and stock solution of HeLa cells was made in HeLa maintenance medium composed of 63.5% HeLa mixture 199, 3% of a 5% sodium bicarbonate solution, 1% of a 20% solution of glucose, 25% tryptose phosphate broth and 7.5% calf serum. (Final pH of this medium was 7.4.*) No antibiotics were added. The cultures were initiated in 15 by 125 mm test tubes having bakelite caps and neoprene liners. Approximately 50,000 cells were suspended in each 1 ml of maintenance medium. Tubes were incubated at 37°C for 72 hours at a slant of 5°. After microscopic examinations were made of the tubes, usable ones were washed twice with phosphate buffered saline and replenished with 1 ml of maintenance medium. In attempting conversion of fungus to tissue phase a small amount of mycelium of each isolate was transferred to 1 ml of HeLa maintenance medium containing HeLa cells. These tubes were incubated, in a slanting position, at 37°C in the roller drum for 7 days. Microscopic examination was made at weekly intervals of growth, to determine degree of conversion. Then 0.1 ml of the culture was transferred, using sterile pipette, to a fresh tube of HeLa maintenance medium containing HeLa cells. This procedure was followed at the end of each week until isolates were completely converted to the yeast phase. The above procedure was followed in another experiment with the exception that the maintenance medium did not contain HeLa cells. It was of interest to learn whether HeLa cells were necessary to maintain *H. capsulatum* in its yeast phase. Accordingly, a series of tubes containing 1 ml of HeLa maintenance medium with HeLa cells and another series without HeLa cells were inoculated with a standard inoculum of yeast cells. The tubes were then placed in the roller drum and periodic read-

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* We are indebted to H. A. Wenner, M.D., for this medium.

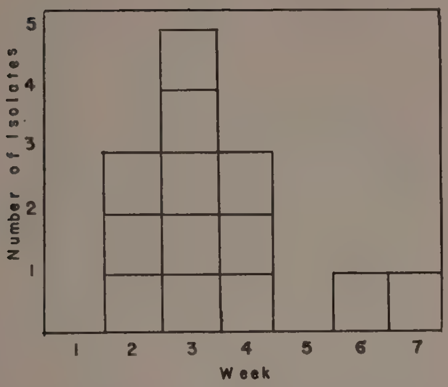

FIG. 1. Conversion of *Histoplasma capsulatum* to the yeast phase in the presence of HeLa cells. Illustrated are the No. of weeks required to convert 13 isolates in tissue culture medium.

ings made to determine amount and rate of growth. In addition to above experiments a study was made to determine the conditions necessary for propagation and maintenance of large numbers of yeast cells. Twenty ml of HeLa maintenance medium were dispensed into a series of sterile 100 ml centrifuge bottles with neoprene stoppers. Five isolates of the fungus previously converted to their yeast phase were selected. Five ml of a standard yeast inoculum were added to duplicate centrifuge bottles of each isolate. One set was placed in the roller drum; the other set remained stationary at 37°C. Daily readings were made to determine rate of growth and hydrogen-ion change of medium. Direct macroscopical observations of bottles were made to determine if rotation was necessary for maximum growth and maintenance of fungus in its yeast phase.

Results. Conversion to yeast phase with HeLa cells: In Fig. 1, the results of the conversion studies are presented. From these results it can be seen that there are differences in rate of conversion in HeLa maintenance medium from the original mycelial phase. At the end of 2 weeks incubation in roller drum, 3 isolates had completely converted to the tissue phase. Examination of several microscopical preparations from these tubes failed to reveal any fragments of mycelium. The greatest number of isolates (five) converted by the end of 3 weeks. Three additional isolates completely converted by the end of four weeks. The twelfth isolate converted by 6 weeks and the thirteenth reached complete conversion by the end of seventh week.

Conversion studies in absence of HeLa cells: Results of attempts at conversion to the yeast phase without HeLa cells are shown in Fig. 2. Six of the 13 isolates successfully converted completely to the yeast phase in the medium without HeLa cells. The first complete transition, 3 isolates, occurred by the end of fourth week. Four additional isolates converted, 2 in the fifth and 2 in sixth week of incubation. Six isolates failed to convert and very few, if any, yeast cells were seen in the microscopical preparation at the end of seventh week, when experiment was terminated.

Maintenance and propagation of yeast phase after conversion in presence and absence of HeLa cells: When the fungus has been converted completely to the yeast phase, HeLa cells are not absolutely necessary for propagation and maintenance of the culture. They do, however, enhance the rate of growth. In 4 of the 5 isolates studied, maximum growth occurred on the fourth day of incubation in medium containing HeLa cells. The

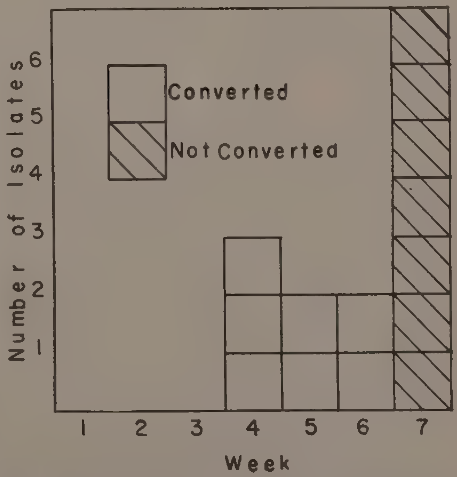

FIG. 2. Conversion of *Histoplasma capsulatum* to the yeast phase without HeLa cells. Illustrated are the weeks of transition for the 7 isolates which converted completely. Six isolates failed to convert by the end of 7 wk.

fifth isolate reached its maximum growth on sixth day.

In the medium without HeLa cells the same 5 isolates required 8 or 9 days to obtain comparable growth as reached in medium containing HeLa cells.

Determination of increase in numbers of cells by volumetric measurements showed an average increase of 30-fold. There was little difference between tubes containing HeLa cells and those without HeLa cells in the medium. In the small test tubes containing 1 ml of medium without HeLa cells there was an indication of reversion, in that a small amount of mycelium was found in 2 of the 5 isolates. During later studies in which large centrifuge bottles containing 20 ml of medium were used, no mycelium was detected in any cultures of the 5 isolates through 10 series of propagations.

Maintenance after conversion with and without rotation: The ultimate use of propagated yeast was to be in antifungal evaluation studies, which required large quantities of yeast cells. The amounts of yeast in small tubes were inadequate for experimentation. The successful conversion and maintenance of the yeast phase in medium without HeLa cells led to studies using 20 ml of medium in 100 ml centrifuge bottles with neoprene stoppers. This was the maximum amount of medium plus the quantity of inoculum that could be used in the slanting position of the roller drum.

The same 5 isolates showed a marked difference in rate of growth in the 100 ml centrifuge bottles when subjected to rotation or held stationary. Four of the 5 isolates reached maximum growth on fourth day of rotation, the fifth isolate on the fifth day. None of the bottles maintained in stationary position in the same incubator reached a comparable growth at end of 10 days.

Discussion. In our experience the tissue culture method has proved very satisfactory for conversion and maintenance of *H. capsulatum* in the yeast phase. It is apparent from Figs. 1 and 2 that presence of HeLa cells in the medium has a marked influence upon rate and final outcome of the yeast phase transition. The ease with which 13 isolates of fungus converted to the tissue phase suggests that the method is rapid and reproducible. Large quantities of cells can be produced by this technic. It has the advantage that the yeast phase can be readily propagated in a liquid medium by weekly transfers. After acclimation to a liquid menstruum and using the roller drum method, these cells can be easily incorporated in antifungal studies.

In addition to *H. capsulatum* this method has proved satisfactory in preliminary studies, for conversion and maintenance of *Blastomyces dermatitidis, Sporotrichum schenckii* and *Coccidioides immitis*. The last named fungus was most difficult to convert to the tissue phase. Data available at this time suggest that the ratio of medium to inoculum is critical. Too much mycelium tends to prevent formation of large numbers of spherules. It is of significance that copious mycelium developed at 37°C when small pieces of infected liver or spleen tissues from mice were inoculated into HeLa maintenance medium. Very few spherules were observed in the first transfer from infected tissue. The ease of mycelium production at blood temperature in a defined liquid medium may be correlated with the frequent reports of mycelium in lungs infected with *C. immitis*.

Candida albicans and *Cryptococcus neoformans* were easily propagated in tissue medium without HeLa cells.

The results of these investigations stimulated further use of tissue culture methods in evaluating antifungal agents against systemic fungi. The technic lends itself very well to evaluation of the effects of the antifungal agent on HeLa cells and tissue phase of the fungus.

It is recognized that tissue culture method is expensive and requires that personnel be proficient in the technic. However, the widespread adoption of the method in virology and other studies has made this laboratory tool available in most research institutions.

Conclusions. 1. Tissue culture method can be successfully used to convert *Histoplasma capsulatum* to the tissue phase. 2. Six of 13 isolations did not convert to the tissue phase in absence of HeLa cells; with all isolates conversion was more complete and rapid in presence of cells. 3. The yeast phase can be propagated and maintained in tissue culture mediums without HeLa cells in 100 ml centrifuge bottles. 4. Rotation of cultures is necessary to assure maximum growth of yeast cells in tissue culture medium. 5. The technic can be successfully used to convert other diphasic systemic fungi, *Blastomyces dermatitidis*, *Sporotrichum schenckii*, and *Coccidioides immitis* to their tissue phase. 6. *Candida albicans* and *Cryptococcus neoformans* can be propagated in tissue culture medium without HeLa cells.

1. Campbell, C. C., *J. Bact.*, 1947, v54, 263.
2. Salvin, S. B., *J. Inf. Dis.*, 1949, v84, 275.
3. Kurung, J. M., and Yegian, D., *Am. J. Cl. Path*, 1954, v24, 74.
4. Littman, M. L., *ibid.*, 1955, v25, 1148.
5. Randall, C. C., and McVickar, D. L., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 150.
6. Randall, C. C., and Hackney, Alice L., *Am. J. Path.*, 1953, v29, 861.
7. Randall, C. C., and Turner, Dorothy J., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 584.

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Effect of *Rauwolfia serpentina* on Thyroid and Pituitary Glands of Young Rats.* (22839)

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The active components of *Rauwolfia serpentina* are held to exert their effects by way of the central nervous system, and it is believed that their influence on the endocrine glands is of minor consequence (Gaunt *et al.* 1). However, in the present study the prolonged administration of a preparation of the whole root to a group of young rats resulted in a significant diminution of the weights of both the pituitary and thyroid glands.

Procedure. The rats employed were of the Sprague-Dawley strain, females, and 21 days of age at the beginning of the experiment. Twenty were treated and 16 littermates remained uninjected as controls. The *Rauwolfia serpentina* was given in aqueous solution by subcutaneous injection and at a daily dosage equivalent to 1 mg per kilo body weight. A fresh solution was prepared and adjusted once weekly to the weight of the animals. The injections were continued until the animals were from 65 to 70 days old when they were sacrificed with illuminating gas. At autopsy the body weight and the weights of the hypophysis, thyroid, adrenals, and ovaries were obtained.

Results. The rats remained active and in good condition throughout the period of treatment, although the injected animals ate somewhat smaller quantities of food and eventually their body weight was generally less than that of the controls. The day of vaginal opening, indicating the onset of complete ovarian function, occurred from the 43rd to the 52nd day of age in the experimental group as compared to the 41st to the 46th day in the controls.

The organ and body weights of the individual rats found at autopsy are given in Table I, while Fig. 1 presents the data for the thyroid and pituitary weights arranged on a semi-logarithmic scale. The oblique lines (broken lines for the controls; continuous for the experimental) represent the average log-organ to body weight relationship as estimated by the method of least squares. The vertical distance between the two lines in each case offers comparison between the organ weights with allowance for body weight. For the pituitary gland this vertical difference is .0338 (in units of logarithms) which corresponds to an 8% smaller size (after adjustment for body weight) in the experimental than in the control group. This dif-

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TABLE I. Body and Organ Weights of Rats following Daily Injection of *Rauwolfia serpentina* 1 mg per Kilo Body Weight for 44 to 49 Days.

Experimental					Controls				
Body wt	Adren.	Ovaries	Thyroid	Pit.	Body wt	Adren.	Ovaries	Thyroid	Pit.
				(g)					
146	.041	.041	.006	.0065	170	.047	.049	.011	.009
168	.045	.056	.0065	.0085	169	.051	.052	.010	"
180	.046	.050	"	"	174	.045	.047	.011	"
157	.039	.053	"	"	184	.059	.046	"	.010
156	.051	.051	"	.008	186	.048	.072	"	.011
171	.054	.056	.007	.0085	188	.053	.063	.010	.010
186	.042	.059	.008	.0095	196	.054	.057	.011	.011
188	.060	.066	.0075	"	201	.058	.069	"	"
153	.041	.048	"	.0075	197	.049	.058	"	"
197	.053	.046	"	.0095	184	.052	.051	"	.010
200	.060	.066	.0085	.010	166	.048	.050	"	"
134	.048	.020	.0065	.0075	180	.054	.046	"	"
180	.045	.055	.008	.0095	170	.048	.024	.010	.008
184	.055	.054	"	"	190	.068	.061	.011	.010
165	.042	.031	.0075	.008	184	.070	.074	"	.011
158	.040	.047	.007	.0075	189	.065	.071	.010	"
190	.051	.054	.008	.0095					
191	.046	.065	"	.010					
188	.055	.062	.009	"					
174	.052	.055	.0085	.009					

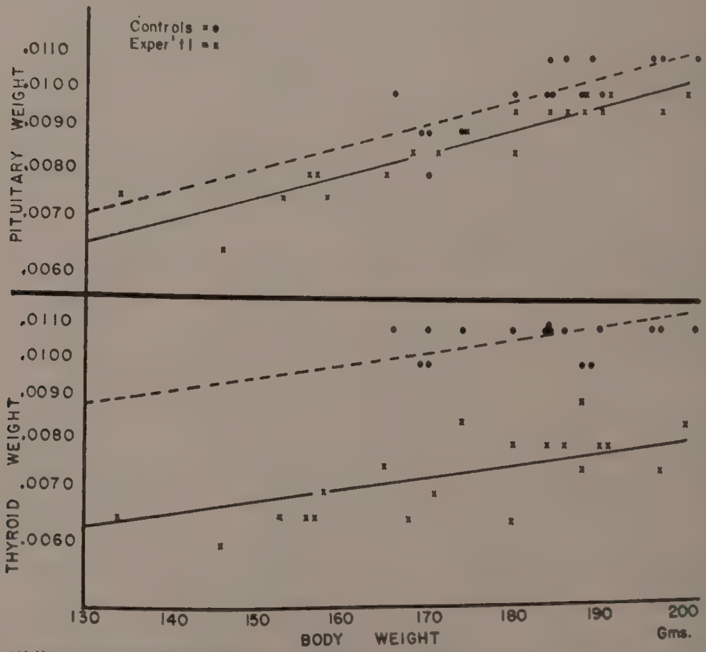

FIG. 1. Oblique lines (broken lines for controls, continuous for experimental) represent avg log-organ to body wt relationship as estimated by the method of least squares.

ference has a standard error of .0126 and is thus statistically significant at the 1% level.

The corresponding difference for the weights of the thyroid is .1467 (standard error of .01), corresponding to a 40% smaller weight in the experimental group than in the control. The data for the ovaries and the adrenals show no effect when analysed statistically by the same method (analysis of covariance).

Administration of *Rauwolfia serpentina* at daily dosages of 2, 3, and 6 mg per kilo body weight and for similar periods of time resulted in such a profound loss of body weight that it was felt that organ differences were greatly affected by changes due solely to partial starvation. Another group of 20 rats was treated in the same manner with a daily dosage of 2.5 mg per kilo body weight of chlorpromazine (Thorazine—S.K.&F.) but without any appreciable change in body and organ weights.

Summary. 1. An extract of *Rauwolfia serpentina* was administered daily by subcutaneous injection to 20 rats. The daily dosage was equivalent to 1 mg/kilo body weight and was continued from 21st to the 65th to 70th days of life. At autopsy body weights and weights of pituitary gland, thyroid, ovaries, and adrenals were obtained. 2. Application of analysis of covariance leads to the conclusion that at this dose level administration of *Rauwolfia serpentina* to young rats resulted in (1) a diminution of weight of thyroid by 40%, at the 1% level; (2) a similar reduction in weight of pituitary gland by 8%; (3) no significant change in either ovarian or adrenal weights, after making allowance for body weight differences.

My thanks are due to Dr. Lincoln E. Moses for statistical analyses; to Mr. V. Montesclaros for technical assistance; to Dr. Georg Cronheim and Riker Laboratories for *Rauwolfia serpentina* and to Dr. William E. Kirsch and Smith, Kline and French for the chlorpromazine.

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1. Gaunt, R., Renzi, A. A., Antonchak, N., Miller, G. J., and Gilman, M., *Ann. N. Y. Acad. Sc.*, 1954, v59, 22

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